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THE EXPONENTIAL DEVELOPMENT OF PENICILLIN RESISTANCE BY NEISSERIA

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It is believed that some evidence can be submitted that the development of penicillin resistance by neisseria is explicable on the basis of Hinshelwood's[1] theory of development of resistance by the expansion of bacterial enzymes. In the case of neisseria the theory is advanced that there is indicated the expansion of a single enzyme or system, or of a substance, for example capsular, produced by such an enzyme. This evidence is based on the observed exponential development of resistance plotted against transfers and represents only one of the possible explanations.

METHODS

The method followed of developing resistance was that of Bahn and associates.[2] Several freshly isolated strains of gonococcus, obtained from the Durham Clinic, were incubated at 36 C under increased tensions of carbon dioxide and water. These latter were obtained by placing a beaker of steaming water as well as the conventional lighted candle in the anaerobic jar. Difco proteose number 3 hemoglobin agar was chosen as the standard basic medium. Subcultures every two or three days were made for several weeks to acclimate the strains to growth on the medium.

Initial penicillin resistances were determined in series of penicillin concentrations in the broth of Bahn and associates with subcultures on chocolate agar to test viability. For three strains the initial resistances determined in this way were 0.005, 0.006, and 0.008 units per ml. Penicillin resistance was increased by passage of the strains through broth cultures containing graded concentrations of penicillin. These gradations consisted of series of random steps of about 10% to 30% of the previously attained resistances.

Frequent subcultures were made on chocolate agar slants for storage at 36 C under sterile mineral oil according to the method of Hac[3] and others. When a strain was lost, it was usually available from an oiled slant at the same or a little lower penicillin resistance level.

RESULTS

After 12 to 15 passages through penicillin medium, resistances had increased about 20 to 70 times, as shown in figure 1. Here the logarithm of penicillin concentration is plotted against the number of passages through penicillin broth after the manner of Miller and Bohnhoff[4] with meningococci.

Another way to express the exponential relationship, comparable to that for the multiplication of bacteria, is $r = A b^p$. Here r equals the concentration of penicillin to which a gonococcus becomes resistant, A equals an earlier concentration to which the gonococcus was resistant, b is a constant within any straight-line segment or period, and p is the number of intervening passages through penicillin medium. From this

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Adapted from a paper submitted to the Faculty of the Graduate School of the University of North Carolina in partial fulfillment of the requirements for the degree of Master of Science.

1. Davies, D. S. and Hinshelwood, C. N. 1943, The adaptation of *Bact. lactis aerogenes* to growth in the presence of sulphonamides. Trans. Faraday Soc. **39**: 431–444.
2. Bahn, J. M., Ackerman, H. and Carpenter, C. M. 1945, Development of in vitro penicillin resistant strains of the gonococcus. Proc. Soc. Exper. Biol. & Med. **58**: 21–24.
3. Hac, L. 1940, Preservation of cultures of *Neisseria gonorrhea*. Proc. Soc. Exper. Biol. & Med. **45**: 381–383.
4. Miller, C. P. and Bohnhoff, M. 1947, Studies on the action of penicillin. VI. Further observations on the development of penicillin resistance by meningococci in vitro. J. Infect. Dis. **81**: 147–156.

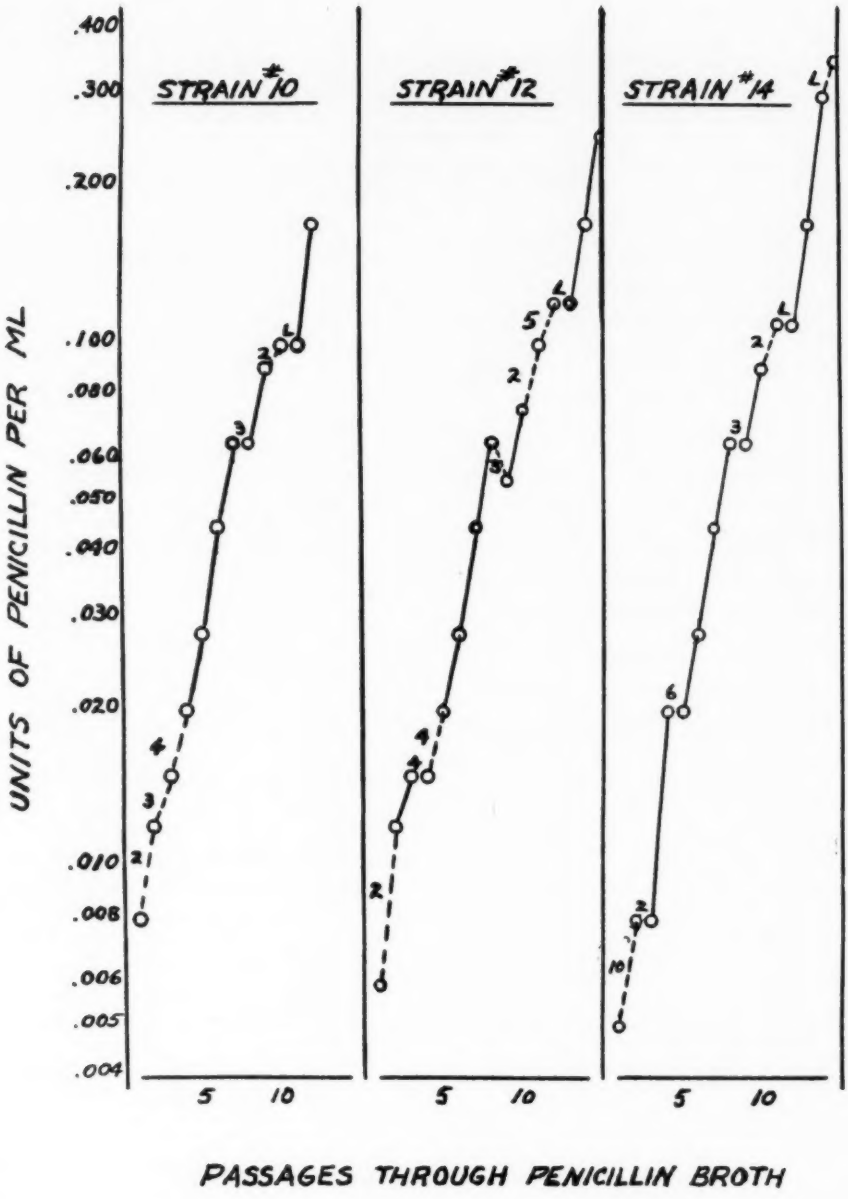

FIG. 1.—Semi-logarithmic plot of development of penicillin resistance in vitro by gonococci.

A dotted line with small number beside it indicates a number of intervening cultures on penicillin-free chocolate agar, from the last of which inoculation into the penicillin broth series in question was made.

L—indicates storage under sterile mineral oil intervened.

the rate of increase with number of passages may be written as

$$\frac{dr}{dp} = Ab^p ln\ b = r\ ln\ b.$$

Also, if $t = kp$, where k is a constant, then $r = Ab^{t/k}$ and

$$\frac{dr}{dt} = \frac{Ab^{t/k} ln\ b}{k} = \frac{r\ ln\ b}{k}.$$

A statistical analysis was made to test the application of the experimental equation, $r = Ab^p$, over larger periods of acquiring resistance than single straight-line segments. From the data for each strain, expressed as logarithms of resistance corresponding to passages through penicillin medium, linear equations of regression and over-all values of "the resistance rate coefficient," $ln\ b$, may be obtained according to the method of Fisher.[5] As illustrated in table 1, this

TABLE 1.—*Illustration of methods for calculating regression coefficients and other parameters.*

n	V	U	U_c	$U(V-\bar{V})$	$(U-U_c)^2$
1	0	0.903	0.822	−4.854	0.00661
2	2	1.079	1.095	−3.642	0.00026
3	3	1.176	1.232	−2.793	0.00315
4	4	1.301	1.369	−1.789	0.00464
5	7	1.813	1.779	2.946	0.00112
6	8	1.954	1.916	5.130	0.00145
7	9	2.000	2.053	7.250	0.00281
8	10	2.230	2.190	10.316	0.00165
8	43	12.456		12.564	0.02169

V is from p; $U = \log 1000r$; $\bar{V} = 43/8 = 5.38$; $D = \frac{12.456}{8} = 1.557$; sum $(V-\bar{V})^2 = 91.88$; $c = \frac{12.564}{91.88}$; $U_c = D + c(V-\bar{V}) = 1.557 + 0.1367\ (V-\bar{V})$; $S_u = \left(\frac{0.02169}{8-2}\right)^{1/2} = 0.0602$; S.E. of $D = \frac{0.0602}{(8)^{1/2}} = 0.0213$; S.E. of $c = \frac{0.0602}{(91.88)^{1/2}} = 0.00628$; t for $c = \frac{0.1367}{0.00628} = 21.77$.

was done for the increase of penicillin resistance of the 3 strains of gonococcus and of 8 strains of meningococcus of Miller and Bohnhoff[4] shown in their figure 2. Calculations were simplified without altering the values of the regression coefficients by multiplying the resistances of the gonococci by 1,000 and of the meningococci by 10. Constants and coefficients of regression. their standard errors, and the student's t's were obtained and may be referred to in table 2. In every case the student's t was greater than the minimum permitted for a probability of 0.01.

TABLE 2.—*Regression coefficients, standard errors, and student t's for equations for increase of penicillin resistance.*

Equation: $U_c = D + c(V-\bar{V})$.

Strain	D	S.E. of D	c*	S.E. of c	t
		For the gonococci:			
10	1.56	0.021	0.137	0.0063	21.8
14	1.77	0.031	0.183	0.0090	20.4
12	1.23	0.027	0.163	0.0130	12.6
	2.05	0.010	0.127	0.0061	20.8
		For the meningococci:			
185	1.11	0.044	0.096	0.011	9.0
299	1.11	0.031	0.096	0.007	13.4
195	.62	0.052	0.174	0.020	8.8
367	1.21	0.035	0.128	0.010	12.3
162	1.17	0.048	0.136	0.017	8.0
81	.91	0.059	0.199	0.024	8.3
380	1.15	0.025	0.095	0.005	19.1
382	1.11	0.040	0.131	0.014	9.6

In this (Fisher's) form of the regression equation the constant, D is the mean value of the dependent variable, U.

* c—over-all value of $\log b = \frac{ln\ b}{2.303}$.

DISCUSSION

One of the attempts to explain the development of resistance, that of Davies and Hinshelwood,[1] postulated for the increase of a cellular enzyme, during certain periods, an exponential equation, $x = x_0 e^{kt}$. The assumption was that the "rate of accretion to the actual substance of any particular enzyme [was] proportional to the amount of it present at a given time," or $dx/dt = kx$. Their basic concept was that of systems of interdependent enzymes, of which one produced a substance required as the substrate by a second. The antibacterial was thought to interfere with the production or use of this substance, or to act directly upon one of the

5. Fisher, R. A. 1946, Statistical Methods for Research Workers, ed. 10, Edinburgh and London, Oliver and Boyd.

enzymes. As for the mechanism of resistance, the results of their experiments seemed to conform best to the concept of the change of the relative activity of alternate enzyme systems of which one was partially blocked by the antibacterial, in their case a sulphonamide.

If, for the moment, an application of this line of reasoning is made to the exponential development of resistance of neisseria, there is indicated the expansion of a single enzyme or single enzyme system. Facts that appear to support this are the high sensitivity of the organisms to penicillin, the relatively slow initial development of resistance, and the conformation of the equation for neisseria with that of the development of a single enzyme or shift of ratio of enzymes within a single enzyme system. This last seems to follow from Davies and Hinshelwood's statement that "if two enzymes are independent and are concerned in alternative, rather than in linked mechanism, their amounts cannot stand in a fixed relation during cell growth, unless the two rate constants are identical, which would not in general be true."

Quite apart from this attempted application of Hinshelwood's theories is the question of the possible significance of the individual resistant rate coefficients, as distinguished from the over-all ones obtained by the statistical method. The fact that identical slopes, and therefore values of the coefficient, recur, sometimes successively, and sometimes apparently at random both for a single strain and for different strains, is believed to be of importance.

When the values of the coefficient were calculated for the gonococci, no relationship within the individual strains was noted. There seemed to be, however, some tendency for the values of the coefficient and also of the increments and decrements between adjacent values of the coefficient within a strain to be squares, divided by 1,000, of relatively small integers. These may be referred to in table 3. If such a tendency could be demonstrated, some conclusions might follow of considerable interest. It might then be indicated, for example, that the maximal rates of acquiring resistance varied with the squares of some fundamental units, such as genetic potentials or with the square of a physical parameter, such as capsular thickness, or its increment.

TABLE 3.—*Resistant rate coefficients within strains of gonococci and the differences between adjacent values.*

Strain 10		Strain 14		Strain 12	
ln b	Difference	*ln b*	Difference	*ln b*	Difference
0.144		0.470		0.693	
0.223	0.079	0.916	0.446	0.223	0.470
0.287	0.064	0.393	0.523	0.287	0.064
0.393	0.106	0.325	0.068	0.393	0.106
0.325	0.068	0.200	0.125	0.310	0.083
0.105	0.220	0.435	0.235	0.287	0.023
0.531	0.426	0.568	0.133	0.182	0.105
		0.154	0.414	0.348	0.166
				0.386	0.038

SUMMARY

1. The penicillin resistances of three strains of gonococci were increased about 20- to 70-fold by passage through series of tubes of broth with increasing concentrations of penicillin.

2. Penicillin resistance of gonococci may be shown to plot exponentially against passages through penicillin medium. Further evidence is added by a statistical analysis.

3. Some adapted theories of Hinshelwood seem to indicate that development of penicillin resistance of neisseria suggests the expansion of a single enzyme rather than replacement of a partially blocked enzyme by another.

4. Only slight evidence was found for a relationship between the individual resistant rate coefficients, or slopes of the exponential curves.

PHYSIOLOGICAL AND PATHOLOGICAL OBSERVATIONS ON FOUR STRAINS OF *TRYPANOSOMA CRUZI*

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Roskin and collaborators (Roskin and Romanova, 1938 and Klyueva and Roskin, 1946) have reported that extracts of *Trypanosoma cruzi* as well as infections with this parasite cure certain types of malignant tumors. Other investigators (Engel, 1944; Hauschka, Saxe and Blair, 1947; and Belkin, Tobie, Kahler and Shear, 1949) could not repeat these experiments successfully, and the suggestion has been made that the use of various strains of the parasite may have influenced the results. It appeared desirable, therefore, to study in some detail whether, or to what extent, different strains of *T. cruzi* possess various qualities.

In the first part of the present paper we examine the question of the influence of 4 strains of *T. cruzi* on the growth rate of rats, their blood sugar and glycogen content, as well as the degree of tissue invasiveness, and the question of the glucose consumption of the bloodstream form of the parasites. In the second part, the glucose consumption, acid and alkali production, and the population growth of the same strains in culture will be discussed.

MATERIALS AND METHODS

Strains of *T. cruzi* used in this comparative study were as follows: The Brazil strain which originated in 1942 from a case of Chagas' disease in Brazil, the Wellcome BH strain which originated in 1926 from a case of Chagas' disease in Brazil (our strain originated from the substrain maintained in mice at the Wellcome Laboratories of Tropical Medicine, London, since 1936), the Guatemala strain isolated a year ago from an infected rat which had been inoculated with flagellate-infected feces from a wild *Rhodnius prolixus* from Guatemala, and the Panama strain which originated in 1935 from a fatal human case in Panama. Each strain has been maintained continuously in white rats by intraperitoneal injections of infected rat blood and in vitro by continuous transfers every two weeks on diphasic blood agar medium (Johnson, 1947). This assured a constant supply of blood and culture forms as needed.

Several litters of rats were employed for the determination of their growth rate, the blood sugar, and the glycogen content for each strain of parasite. Approximately the same number of females and males were infected or remained uninfected as controls, respectively. The experiments were begun when the rats were 8 days old. The infections were achieved by intraperitoneal injection of infected blood. At designated intervals thereafter equal numbers of infected and control litter mates were killed by decapitation. Blood was collected in a heparinized vessel for the blood sugar determinations and for estimating the degree of infection. The body of the rat was then rapidly cut into pieces and immersed into hot 60% KOH. The glycogen was determined according to Pflueger's (1905) method in an aliquot of the dissolved rat tissues; the reducing sugar obtained after inversion was determined by means of Hagedorn and Jensen's (1923) method. The latter method served also for the blood sugar determinations. Duplicate determinations were carried out in every case. The methods employed for measuring the glycolysis and oxygen consumption of the uninfected and infected rat blood have been described previously (von Brand and Tobie, 1948).

For the study of tissue invasiveness, sections of lung, heart, liver, spleen, kidney, muscle, and brain, stained by means of the Lillie (1948) azure A-B eosinate plasma stain and the van Gieson connective tissue stain, were used from rats killed after varying post infection intervals. In some cases sections of bone marrow, adrenal gland, spinal cord, pancreas, and intestine were also examined. All sections were prepared from formalin-fixed organs.

The culture flagellates were grown in the same

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* With the technical assistance of Bruce P. Phillips, Vernal W. Bassett, and Benjamin Mehlman.

wholly liquid medium used during our previous studies (von Brand, Johnson and Rees, 1946 and von Brand and Johnson, 1947), with the difference that in some of the series the amount of sugar added to the Locke's solution serving for the extraction of the blood agar base was varied. Twenty cc of fluid were used for each culture; the inoculation consisted of between 0.5 and 2 million organisms per cc. The cultures were incubated at 24 C. For each strain from 2 to 4 cultures were set up in each series, and most series were repeated at least twice. Immediately after inoculation, 2 cc amounts were withdrawn from each culture. They served for the sugar determinations (duplicate determinations according to Hagedorn and Jensen's [1923] method before and after yeast digestion), the pH determinations (Beckman glass electrode apparatus), and the counts (Levy counting chamber) of organisms. At designated intervals after inoculation samples from 1 to 4 cultures were analyzed in the same way for each strain. Throughout the experiments strict aseptic precautions were maintained, and any culture showing contamination was immediately discarded.

Growth retardation, tissue invasiveness, and the number of flagellates appearing in the peripheral circulation appeared to be related to pathogenicity. An early and severe stunting of growth occurred in the Brazil strain, the infected animals weighing only 67% of the uninfected ones after three weeks. Progressively less influence on growth was shown by the Guatemala, Wellcome, and Panama strains, in that order, the latter showing no measurable growth retardation at all.

Parasitization of tissues.—The Brazil strain exhibited also the highest degree of tissue invasiveness (table 1), followed in decreasing order by the Guatemala, Panama, and Wellcome strains. In addition to the organs shown in table 1 which were studied for every animal, the adrenals, intestine, bone marrow, and spinal cord of a few animals were used. In these latter organs parasites were found only in the adrenals and the intestine of rats infected with the Brazil strain. Concerning histological details, it may be mentioned that both the Brazil and Guatemala strains showed invasion of the bronchial smooth musculature and of the smooth musculature of vascular walls in several instances. In sections of the intestine smooth muscle was also involved. In the brain the organisms appeared to parasitize glia cells. Occasional organisms were observed in von Kupffer cells, and in some instances a few organisms were seen in the vicinity of histiocytic aggregates as described by Mazza and Jörg (1934). The organisms found in the spleen appeared swollen

TABLE 1.—*Tissue invasiveness of four strains of* Trypanosoma cruzi.

Strain	Number of animals	Age of infection	Number of animals with parasites in designated tissue						
			Heart	Lung	Liver	Spleen	Kidney	Muscle	Brain
		Days							
Brazil	6	10–24	6	4	4	4	0	5	3
Guatemala	7	10–28	7	2	0	1	0	6	0
Wellcome	10	10–31	5	0	0	0	0	1	0
Panama	5	15–29	5	0	1	0	0	1	0

RESULTS

Animal experiments

The pathogenicity of the 4 strains was noticeably different. The Brazil strain killed virtually all rats during the third week of infection. The Guatemala strain did not kill them at the time the experiments were carried out, but a few months later its virulence had increased to such an extent that many of the rats then died 3 to 4 weeks after inoculation. Infections with the Wellcome and Panama strains were never fatal to rats at the time of the experiments, nor are they now. The former does rapidly kill mice; the other strains have not been tested by us in this respect. Neither of the strains kills guinea pigs.

and were apparently contained in phagocytes.

Parasites in blood stream.—The 4 strains differed markedly in the number of trypanosomes appearing in the peripheral blood. The highest counts, ranging up to 150 million per cc, were found in the Brazil strain, followed by the Guatemala strain with a maximum of 45 million per cc, the Wellcome strain with a maximum of 18 million per cc, and the Panama strain, where the parasites never reached higher numbers than about 1 million per cc.

Tissue lesions—The Brazil strain also produced the most severe changes in the tissues of the infected rats. In infections with this strain the cells of the alveolar septa of the lung showed a progressive degree of proliferation and hypertrophy, the maximum degree of change being present in the rat killed on the 16th day following infection. At 10 and 11 days there was an increased number of cells lining the alveolar walls, but the nuclei still were flat. By the 14th day these cells had assumed a cuboidal shape with the nuclei becoming ovoid and the cytoplasm abundant. In several areas stratification of these cells was apparent, and by the 16th day many of the alveoli appeared almost completely occluded by this hypertrophied tissue. The two rats killed on the 21st and 24th post infection days showed less severe changes than the animal killed at 16 days. Throughout the entire course of the disease there was a diffuse infiltration of the lung with lymphocytes.

The heart was the most severely affected tissue. The interstitial tissue of the myocardium in the early stages was edematous and infiltrated irregularly with lymphocytes and undifferentiated mononuclear cells. At 11 to 14 days fibroblasts were also present. The amount of interstitium increased throughout the course of the disease, and by 21 to 24 days post infection the muscle fibers were widely separated. The entire muscular wall of the heart was diffusely infiltrated with lymphocytes. The epicardium and endocardium, including the atrio-ventricular valves, showed a lymphocytic infiltration in the later stages of the disease.

Sections of skeletal muscle showed lesions less severe but similar to those observed in heart muscle.

The liver showed fatty degeneration and small focal accumulations of lymphocytes lying in the sinusoids in the midlobular regions.

The lymphoid follicles of the spleen showed hyperplasia without a loss of the normal architecture. The reticular phagocytes were vacuolated and contained excessive amounts of blood pigment, and the entire spleen appeared congested.

There were inconstant changes in other tissues. In several of the animals there were small fibrotic foci in the cortex of the kidney but otherwise no significant lesions. Occasional small foci of reacting microglia were observed in the cerebral cortex and hippocampus. These cells were not always associated with parasites. A few parasites occurred extracellularly in the perivascular spaces. The muscularis of the small intestine in a few of the animals was infiltrated with mononuclear cells. No pathology was observed in the pancreas, adrenals, or bone marrow.

The Guatemala strain produced similar lesions of the lungs and heart but was slower in reaching the same degree of severity seen with the Brazil strain. The type of change produced in the lung by the latter strain at 16 days was not observed until 21 days in rats infected with the Guatemala strain. The severity of the cardiac and hepatic lesions more closely paralleled those seen in the Brazil strain infected animals. No pathology

was observed in the brain, kidneys, intestine, adrenals, pancreas, or bone marrow.

The Wellcome strain was rather irregular in its pathogenicity, in some cases producing lesions as severe as those seen with the Brazil strain, while some of the rats showed minimal changes in all tissues.

Only 2 out of 5 rats infected with the Panama strain showed significant heart and lung lesions, and these were less severe than those produced by the other three strains. No significant changes were observed in any of the other tissues examined except for a mild lymphocytic infiltration of the liver.

The Brazil and Wellcome strains produced no significant lesions in the two guinea-pigs inoculated with each strain. The Guatemala strain produced mild changes in the lungs consisting of increased cellularity of the alveolar capillaries and diffuse lymphocytic infiltration. No pathology was present in other tissues examined.

Blood sugar and glycogen.—The blood sugar and total glycogen showed no pronounced differences in cases of infection with the different strains. In order to test whether the infection had a measurable effect, the entire material for the 4 strains was averaged (table 2). A comparison with the values obtained from the non-infected controls shows that the infection has no significant influence on either blood sugar or glycogen content.

TABLE 2.—*Mean values of the blood sugar and glycogen content of normal rats and rats infected with* Trypanosoma cruzi.

Days*	Blood sugar, mg percent		Glycogen, percent	
	Uninfected	Infected	Uninfected	Infected
0	139±2.5		0.26±0.009	
7	146±2.2	145±1.9	0.29±0.020	0.27±0.019
14	148±3.0	140±3.0	0.34±0.017	0.27±0.014
21	139±2.1	127±3.3	0.36±0.027	0.31±0.021
28	130±1.8	132±2.8	0.39±0.022	0.49±0.031

* 0 days corresponds to the day on which groups of rat were infected.

Sugar consumption of parasites.—An attempt to determine the rate of sugar consumption of the blood-stream form of *T. cruzi* (Brazil strain) in vitro failed, despite variations of incubation time from 1½ to 22 hours and variations in the initial sugar content from 141 to 419 mg per cent. In every case, as much sugar disappeared from normal control blood as from the infected samples. This is true also for one series where the oxygen consumption of the parasites was determined and found to correspond to 78 mm^3. In order to test whether a decrease in formed blood elements may have decreased the glycolysis of infected blood and may thus have masked a sugar consumption by the parasites, red and white cell counts were carried out on 8 young non-infected rats and 8 infected litter mates at the height of the infection. No difference was found, and thus we have concluded that the rate of sugar consumption of the blood form of *T. cruzi* is extremely low, if it consumes sugar at all.

Experiments with cultures

During the extraction of the blood agar bases with Locke's solution prior to the setting up of the actual experiments, a rather considerable amount of nonfermentable reducing material diffused into the overlay. The glucose fraction was separated therefrom during the experiments by determining in each sample the total reducing substances and the nonfermentable reducing substances. The latter remained, within reasonable

limits of error, constant in a given series, but varied somewhat from series to series. At the conclusion of each series, the values for the nonfermentable substances found in the various samples were averaged and subtracted from the individual determinations of the total reducing substances; the difference represented the glucose values. This correction varied in the different series from 75 to 112 mg per cent.

Conversely, large amounts of glucose diffused from the Locke's solution into the base. The amounts of sugar lost in this way varied again in the different series and were not dependent only on the amount of glucose present in the overlay. Small differences in the consistency of the base, for example, may have had an influence on the rate of diffusion. This initial loss of sugar is the reason why the initial glucose values in the series utilizing different strains were of the same order of magnitude but not exactly identical. Four glucose levels were employed, the initial values varying respectively between 25 and 45, 90 and 130, 170 and 195, and 290 and 350 mg per cent.

The results of these experiments are summarized in figures 1 to 4. It is evident that the flagellates were unable to utilize glucose when its concentration was below 40 to 50 mg per cent, regardless of whether this was the original level (series A), or whether it was reached at some time after the start of the experiments due to the sugar consumption of the organisms (series B and C). In series D, which had a higher initial glucose content than the other series, the sugar level remained steady, within the limits of experimental error, at about 80 mg per cent.

Clear connections between sugar consumption and pH of the medium were observed. In series A no acidification whatever occurred, but an early and strong alkalinization took place. In series B and C the medium became first acid and then alkaline; the acidification was more pronounced and protracted in the series with the higher sugar content (series C). In series D a very strong acidification finally took place and the medium at no stage became alkaline.

The development of the population was rather variable even in different flasks of one series with one strain. An analysis of our fairly extensive material gives the following picture. In series A, where the sugar level was so low that it could not be utilized, only a moderate multiplication occurred; the average maximum number was about 25 million organisms per cc. In series B an average of about 80 million per cc was reached at a time when the sugar consumption had practically ceased and the pH was reaching values of around 7.6. In series C an average peak of about 60 million per cc was reached much earlier, the maximum corresponding to a pH value of around 6.0. With increasing acidity the population then declined, but two of the strains (Wellcome and Panama) were capable of building up a new peak of about 80 million per cc when the medium became neutralized, while in the Brazil and Guatemala strains this second peak was not discernible. In series D the population increased rapidly to 30 to 50 million per cc. It then declined and stayed low as long as the experiments lasted, at which time the flagellates showed distinct signs of degeneration.

DISCUSSION

The 4 strains of *T. cruzi* used in the present study differed markedly in their pathogenicity for young rats. A good correlation existed between the degree of tissue invasiveness and the numbers of parasites appearing in the peripheral blood. The question why strains of this

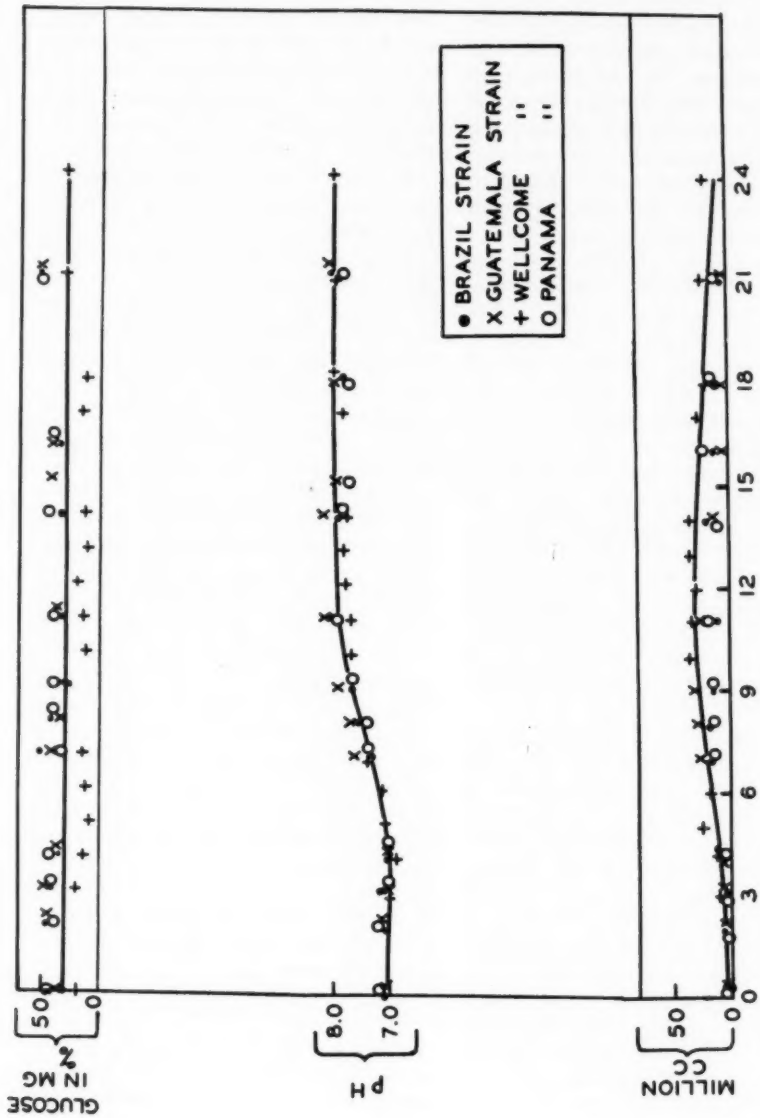

FIG. 1.—Series A: Glucose, p_H, and population in cultures of 4 strains of *T. cruzi*, initial glucose level 25 to 45 mg percent. Incubation temperature 24 C.

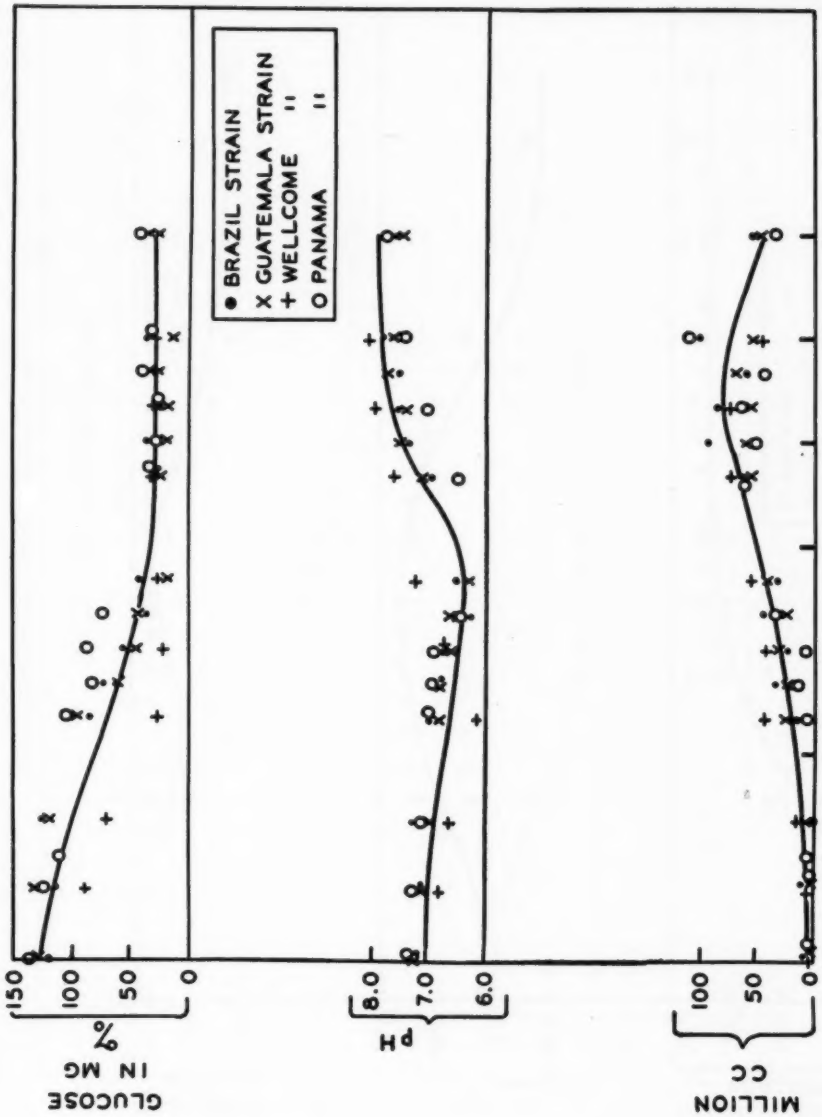

FIG. 2.—Series B: Glucose, p_H, and population in cultures of 4 strains of *T. cruzi*, initial glucose level 90 to 130 mg percent. Incubation temperature 24 C.

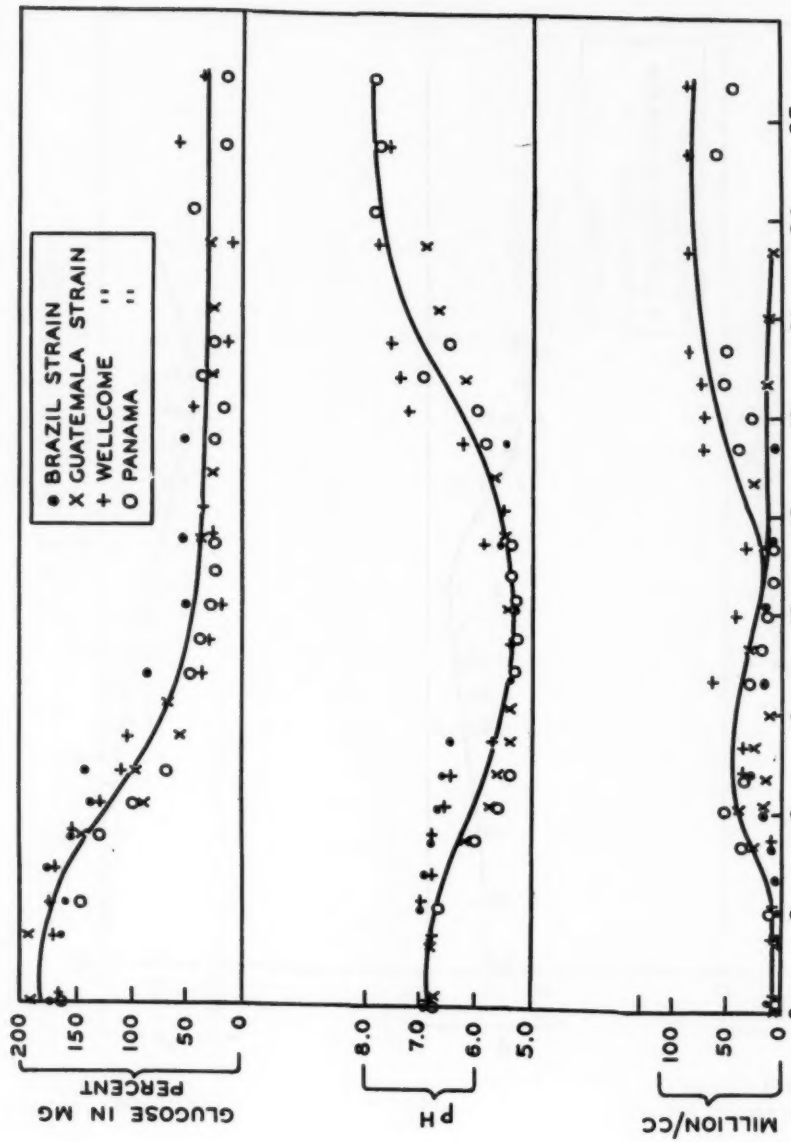

FIG. 3.—Series C: Glucose, *pH*, and population in cultures of 4 strains of *T. cruzi*, initial glucose level 170 to 195 mg percent. Incubation temperature 24 C.

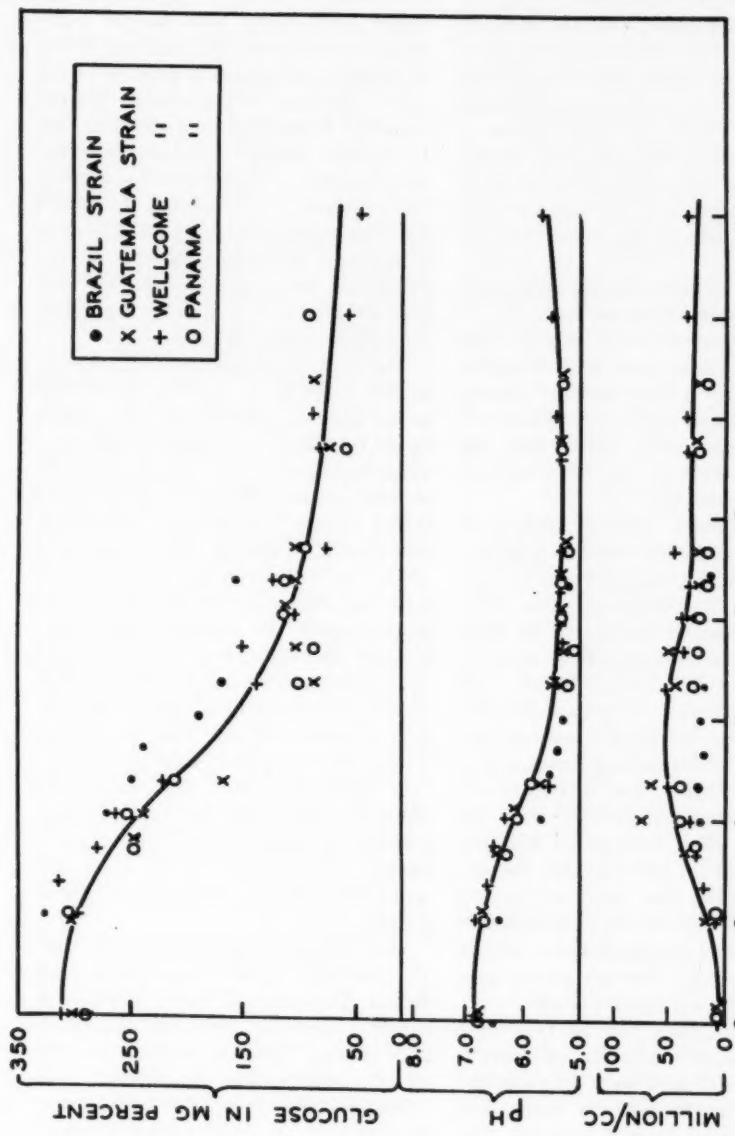

FIG. 4.—Series D: Glucose, p_H, and population in cultures of 4 strains of *T. cruzi*, initial glucose level 290 to 350 mg percent. Incubation temperature 24 C.

species differ in their pathogenicity has been discussed by Reichenow (1934). He considered it possible that the deciding factors lie in the previous history of the parasites, that is, whether a regular alternation between insect and vertebrate occurred or whether a large number of insect passages took place before the vertebrate became infected. In the latter case the virulence would decrease with increasing length of development in the insect.

A certain influence of the insect passage has been demonstrated experimentally. Brumpt (1913) showed that this restores the original level of pathogenicity after it had become altered through experimental vertebrate to vertebrate passages, a view that has been corroborated by Reichenow's (1934) experiments.

If Reichenow's view is correct, it certainly implies that very long lasting modifications are brought about by consecutive insect passages and that these are not necessarily duplicated by completion of the developmental cycle in culture. Our Wellcome, Brazil, and Panama strains are all old strains that have been kept for years in animals and in culture without passing through triatomes. We found in these strains, nevertheless, extreme in pathogenicity for young rats. The findings of Campos (1925) should be noted in this connection. He showed that a strain regularly paralyzing dogs, that is, a neurotropic strain, retained its peculiarities despite frequent passages through guinea pigs and 23 passages through culture.

Our experiments demonstrate that the blood sugar and glycogen content of the infected rats were but slightly lower than in the controls during the second and third week of infection. The values for the blood sugar are still within the normal range and even considerably higher than those found in healthy rats starved for 18 hours, where an average value of 92 mg per cent was found (von Brand and Krogh, 1935) with the same analytical methods. While no similar comparison is possible in the case of glycogen, it is probable that the situation is not fundamentally different. It appears likely, therefore, that the carbohydrate relationships in infected rats are more or less normal. The small lowering of the blood sugar and glycogen values can without difficulty be explained on the assumption that the sick animals consume somewhat less food than their healthy litter mates.

The sugar consumption of the bloodstream form of *T. cruzi* is apparently so low that we were not able to demonstrate it; this is in agreement with previous experience (von Brand, 1933). It should be noted that in the same experiments in which an identical glycolysis was found in infected and uninfected blood, an oxygen consumption of 78 mm^3 was observed that was due to the trypanosomes. In working with parasites of the *evansi*, *brucei* or *congolense* subgroups, such an oxygen consumption would correspond to a sugar consumption of about 0.6 mg (von Brand and Tobie, 1948). A glycolysis of this magnitude would have been detected by the methods used, and we are therefore justified in stating that the sugar—oxygen relationships of *T. cruzi* are quite different from those of the above groups.

Our culture experiments prove that the previously made assumption (von Brand, Johnson and Rees, 1946) of a causal connection between disappearance of sugar from the medium and the latter's acidification is correct. This follows clearly from the observation that no acid production could be demonstrated in a medium containing so little sugar as to be not utilizable by the flagellates and by the observation that

the acidification became more pronounced the more glucose was present initially.

It is probable that the acid production may, in some of our series, have been the limiting factor for the multiplication of the flagellates. In the series with the highest initial glucose content the reaction fell to a pH of about 5.2 and the cultures declined steadily. In some series with medium sugar content the population reached a second peak after the pH began to increase from a low point of about 5.3. A similar development of the population has already been observed by Chang and Negherbon (1947) but they did not study any chemical factor with which it would be correlated.

In a recent paper Chang (1948) states that this second peak in population is due to the development of slender crithidia and trypanosome forms which are able to consume the acids produced from sugar by the leishmanias and oak-leaf-like crithidia forms present in the earlier stages of the cultures. We consider it as likely that a transition to a protein metabolism also takes place. The mere consumption of previously produced acids could hardly account for the pH rise above the neutral point. The fact that a similar pH rise occurs in cultures with negligible initial sugar content indicates that some basic substance, perhaps ammonia, is actually produced.

It is noteworthy that in many of our series the highest numbers of flagellates were encountered when the sugar consumption had ceased due to exhaustion of the available glucose. This does not mean, however, that the culture forms of *T. cruzi* do not require sugar. In our series with the lowest sugar level only a very moderate multiplication took place and the population was never built up to a high level. It would appear then that glucose gives the organisms an initial stimulus, but that they can still divide several times when the sugar becomes exhausted. Whether the flagellates store carbohydrate in their body which may be used during this period would be interesting to investigate.

The values found for sugar consumption, pH, and population growth of the 4 strains fluctuated around the curves drawn in figures 1 to 4. We do not believe that the deviations shown are indicative of constant differences in metabolism between the 4 strains studied. It should be emphasized that in cases where more than one strain was studied at the same time in mediums containing an identical amount of sugar, the various values found for the different strains coincided fairly closely. The differences were much greater when the strains were investigated at different times, but similar variations were encountered when one strain was studied repeatedly at one sugar level. It is reasonable to assume that such variations were due either to differences in the medium (for example, varying content of unidentified growth-promoting substances in the blood used for the bases that were extracted) or to differences in the stock tubes used for the inoculum, rather than to differences inherent in the strains. Chang and Negherbon (1947) studied in a different medium the growth rate of two strains of *T. cruzi;* they, too, reached the conclusion that both reacted identically.

SUMMARY

1. Four strains of *T. cruzi* showed marked differences in pathogenicity for young rats, as indicated by their influence on the growth rate of the host, the degree of tissue invasiveness, and the numbers of flagellates appearing in the peripheral circulation.

2. None of the strains had a pronounced effect on the blood sugar and

the glycogen content of the host.

3. The sugar consumption of the blood stream form of *T. cruzi* is at most very low; parasitized and nonparasitized blood gave identical glycolysis values, despite the fact that the oxygen consumption of the infected blood was much higher than that of the controls due to the respiration of the parasites.

4. Culture forms consumed sugar leading to an acidification of the medium which in cases of high initial glucose values became the limiting factor for the multiplication of the parasites. In mediums with medium sugar content, the acidification was followed by an alkalinization; the population peak was in most of these cases reached when the medium had again become neutralized. When the initial glucose content was so low as not to be utilizable, no acidification but only alkalinization occurred; the flagellates then multiplied only to a moderate extent.

5. No distinct differences in the cultural behavior between the 4 strains could be observed.

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DIPHTHERIA ANTITOXIN ESTIMATIONS IN THE BLOOD OF THE ADULT POPULATION OF BERGEN, NORWAY, JUST AFTER A DIPHTHERIA EPIDEMIC

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After the last diphtheria epidemic in Bergen had lasted three years, 3000 persons who had not been vaccinated against diphtheria were given the Schick test. Sixty-seven percent proved to be Schick-positive,[1] which was a remarkably high figure so late in the epidemic compared with findings reported elsewhere.[2,3] The ages of those vaccinated ranged from 16 upwards, two-thirds being between 21 and 50 years.

This investigation was extended between December, 1947 and May, 1948 by measuring the diphtheria antitoxin content in the population of Bergen with a view to determining the antitoxin titer of (1) the adult population of Bergen, (2) persons who had suffered from diphtheria earlier and (3) persons vaccinated against diphtheria.

Epidemiological background in Bergen

Between World War I and World War II there was a slow and uniform decline of diphtheria in Bergen. In 1919 the rate was 8.73 per 1000 inhabitants. This figure had declined to 3.71 in 1931 and to 1.45 in 1935. The decline continued during the following years with the lowest level (0.02) in 1940. The diphtheria mortality was 0.46 per 1000 inhabitants in 1919 and had decreased to 0.09 in 1931. In the period from 1935 through 1940 there was not a single death from diphtheria in Bergen.

In Germany during the years preceding World War II, there were extensive and malignant epidemics of diphtheria in which most of the deaths were due to *typus gravis*.[4] It was therefore feared that a diphtheria epidemic would break out in Bergen after it was occupied by the Germans in April, 1940. The number of cases of diphtheria did not rise, however, until the end of 1941. During the following years, it assumed epidemic proportions with a peak of 4.94 per 1000 in 1943 and of 4.77 in 1944. Since then there has been a gradual decline until only 1 case per 1000 was reported in 1947.

During the years preceding this epidemic, *typus mitis* was the predominant type in Bergen, and *typus gravis* had not once been found.[5] During the epidemic *typus mitis* maintained on the whole the same frequency, but *typus intermedius*, which had been the predominant type at the beginning of the epidemic, was gradually replaced by *typus gravis*, which in the first half of 1948 was the only type to be found.[6]

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TABLE 1.—*Diphtheria antitoxin estimations in the blood of unvaccinated persons.*

Units/ml	Years																				Total	
	≤15		16–20		21–25		26–30		31–40		41–50		51–60		61–70		<70				♂	♀
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀				
1		1		1		1	1	1	4	2	2	5	5	3	5			2	34	15	19	15
1				2	5	1	3	3	3	4	8	8	12	10	18	12	8	7	105	48	57	48
0.1		1	1		2	3	2	6	11	9	15	11	20	14	24	22	7	11	158	76	82	76
0.01	1	1	2		4	6	8	9	5	10	8	7	11	7	9	11	2	1	102	52	50	52
0.001	5	6	3	7	11	18	13	29	26	45	22	34	24	22	21	13	10	8	317	182	135	182
Individual total	6	8	6	10	22	29	29	48	49	70	55	65	72	56	77	58	27	29	716		343	373
Combined total	14		16		51		66		119		120		128		135		56					

This type revolution occurred after the epidemic had reached its peak. With the growing prevalence of *typus gravis*, the severity of the disease increased. A mortality of 2.4% in the period from 1941 through 1943 rose to 5.8% in the period from 1944 through 1946.

In 1942 compulsory diphtheria immunization was carried out on the children in the elementary school in Bergen and has been continued. It has also been somewhat extended to other age groups. By the end of 1946, 16,698 of Bergen's 110,000 inhabitants had been immunized against diphtheria with three injections and 8605 with two injections of diphtheria toxoid.

During the epidemic there was a considerable shift in the age distribution of diphtheria to higher age groups. While in 1942, 13% of the patients were over the age of 30, this figure increased to 35% by 1947. In some cases several elderly folk developed diphtheria of a severe character.

Materials and methods

The material consisted of serums from 1000 patients admitted to the Bergen City Hospital, Departments of Medicine, Surgery and Infectious Diseases. None of the patients harbored *Corynebacterium diphtheriae* in the throat or nose.

Both diphtheria standard serum and diphtheria standard toxin were provided by the Department of Biological Standards, The State Serum Institute, Copenhagen.

The antitoxin content of the specimens received was determined by the intracutaneous rabbit method described by Jensen.[7]

7. Jensen, C. 1933, Die intrakutane Kaninchenmethode zur Auswertung von Diphtherie-Toxin und Antitoxin, Copenhagen.

Distribution of antitoxin in the unvaccinated population of Bergen

Among the 1000 persons included in this investigation there were 716 who showed no clinical sign of diphtheria and had not been vaccinated against it. Their sex, age and antitoxin distribution are presented in table 1 (see also fig. 1).

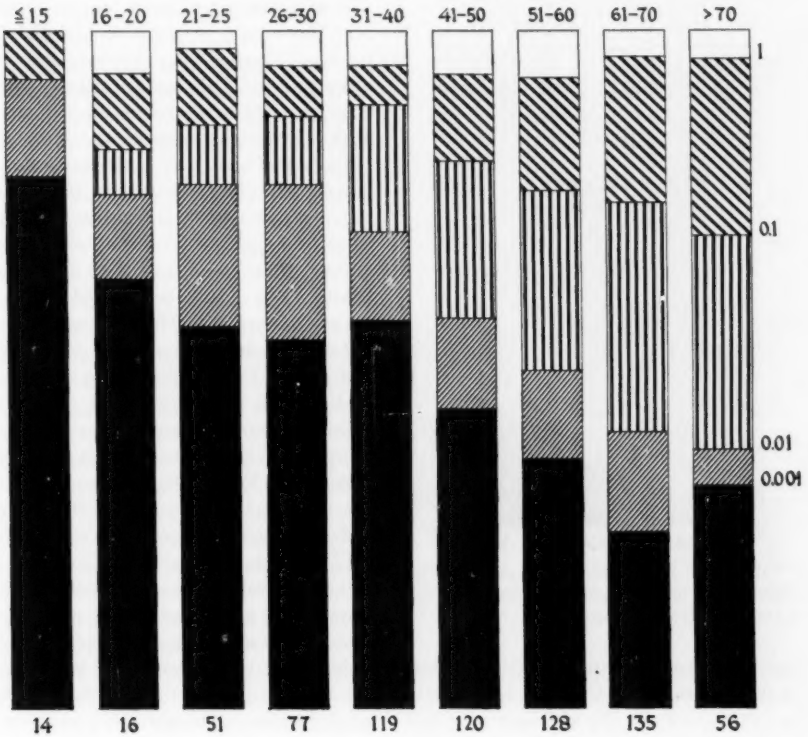

FIG. 1.—Diphtheria antitoxin spectrum of the unvaccinated populations of Bergen.

From this table it can be seen that the antitoxin content of the blood in men and women rose uniformly until the age of 60. Of these 716 persons, 419 (59%) had an antitoxin content of 0.01 units /ml or less. Of the 158 persons within this group under the age of 30, 88% had an antitoxin content of 0.01 units/ml or less and 58% had 0.001 units/ml or less.[8,9,10] In persons over the age of 30 the figures were 296 out of 558 (53%) and 225 out of 558 (40%), respectively. Thus, even after the age of 30, a great proportion of the population of Bergen is susceptible to diphtheria.

Among the 716 persons, 373 (52%) were females. While the males consti-

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tuted the majority who had more than 0.01 units/ml (158 out of 297, i.e., 53%), the females were in the majority among those with an antitoxin content of 0.01 units/ml or less (234 out of 419, i.e., 56%). This sex difference is not great but tallies with the fact that more females than males contract clinically demonstrable diphtheria.

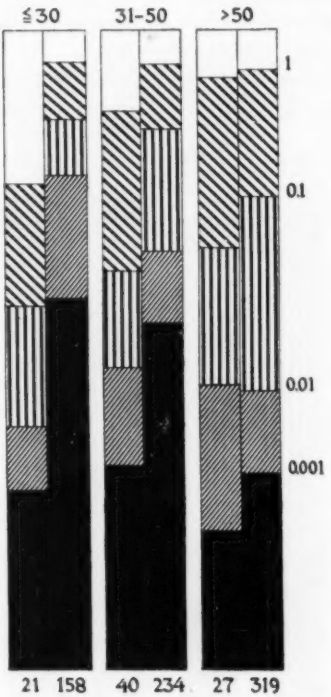

FIG. 2.—A comparison of the antitoxin distribution in unvaccinated persons with and without previous diphtheria.

Antitoxin distribution among unvaccinated persons who had suffered from diphtheria earlier

Among the 1000 persons examined, 96 had suffered from diphtheria at an earlier date although the time of infection was sometimes uncertain. As 4 men and 4 women among the 96 had already been vaccinated with diphtheria toxoid after suffering from diphtheria, there were only 88 nonvaccinated persons with a history of diphtheria.

On the whole, the antitoxin content was found to be somewhat higher among the persons who had contracted clinically demonstrable diphtheria than among those who had not (table 2). While 59% of the latter had an antitoxin content of 0.01 units/ml or less, the corresponding figure for those who had suffered from diphtheria was 44% of 88. This difference was, however, greatest among young persons with a comparatively recent attack of diphtheria (fig. 2). Among persons over the age of 30 whose attacks of diphtheria had presumably occurred years ago, 46% of 67 had an antitoxin content of 0.01 units/ml or less, whereas the corresponding figure for those without a clinical history of diphtheria was 53%. This slight difference may have been due to chance. The finding that no difference exists in the antitoxin content of persons who had had diphtheria long

TABLE 2.—*Diphtheria antitoxin estimations in the blood of unvaccinated persons who had previously had clinical diphtheria.*

Units/ml	Years						Total	♂	♀
	≦30		31–50		>50				
	♂	♀	♂	♀	♂	♀			
>1	2	3	1	4		2	12	3	9
≦1	1	3	4	6	2	5	21	7	14
≦0.1	2	2	2	4	2	4	16	6	10
≦0.01		2	1	5	3	3	14	4	10
≦0.001	2	4	6	7	2	4	25	10	15
Individual total	7	14	14	26	9	18	88	30	58
Combined total	21		40		27				

ago and those who had not agrees with the observation that adult diphtheria patients not infrequently state that they have previously suffered from clinically demonstrable diphtheria.

Antitoxin distribution among persons vaccinated with diphtheria toxoid

None of the persons examined had been vaccinated with diphtheria toxoid before 1942. Among the 196 persons dealt with in this investigation, 162 received three injections each, with an interval of a month between the first and second injection and a year between the second and third injection, and the remaining 34 persons received two injections. Purified ultra-filtrated diphtheria toxoid to which 10% $Al(OH)_3$ had been added was given in doses of 50 L_f per injection to children and half this amount to adults. Among those vaccinated, 8 persons had suffered from diphtheria earlier. They were included among the vaccinated, for, as already pointed out, diphtheria induces no great permanent rise of antitoxin formation.

Among the 196 persons mentioned above, 61 were over the age of 30 (table 3). Most of them had been vaccinated in 1944 or 1945 as a consequence of Schick-testing which had shown them to be Schick-positive. They could therefore be counted on as having an antitoxin content in the blood of 0.01 units/ml or less before vaccination was started.

It will be seen that the antitoxin content of the vaccinated persons was on the whole high. As many as 93% of 196 persons had a titer of more than 0.01 units/ml and 71% had a titer of over 0.1 units/ml. The antitoxin content was practically the same for the two sexes.

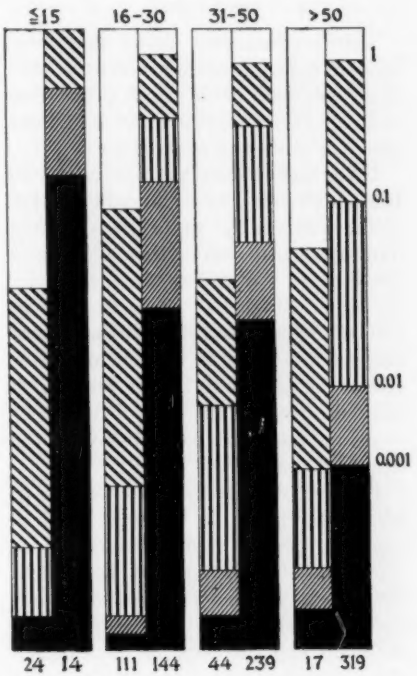

FIG. 3.—A comparison of the antitoxin distribution of vaccinated and unvaccinated persons.

While the antitoxin content of the unvaccinated persons increased with

TABLE 3.—*Diphtheria antitoxin estimations in the blood of vaccinated persons.*

Units/ml	Years ≦15 ♂	≦15 ♀	16–30 ♂	16–30 ♀	31–50 ♂	31–50 ♀	>50 ♂	>50 ♀	Total	♂	♀
>1	6	4	15	16	12	6	2	4	65	35	30
≦1	5	5	26	24	4	5	1	5	75	36	39
≦0.1	1	2	9	16	8	4	1	2	43	19	24
≦0.01			1	2	2	1		1	7	3	4
≦0.001		1	1	1	1	1	1		6	3	3
Individual total	12	12	52	59	27	17	5	12	196	96	100
Combined total	24		111		44		17				

age, that of the vaccinated persons remained the same in the various age groups (fig. 3).

DISCUSSION

This investigation shows that even after an epidemic of diphtheria the antitoxin spectrum of the adult population of Bergen is low compared with findings recorded in several other places.

Gröer and Kassowitz,[2] who examined 1062 children in Vienna, found that 84% of them at the age of 15–18 years had diphtheria antitoxin in their blood in a concentration of 1/200 antitoxin units or more. Zingher's[3] analysis of over 150,000 Schick tests showed that about one-half the children were Schick-positive at the age of 6 to 7 years, one-fourth were positive at the age of 12 to 13 years and one-tenth were positive at the age of 30 years.

In large, densely populated towns in which diphtheria is endemic, Schick-negative persons are relatively more numerous than in sparsely populated, rural areas. Zingher's figures cover several different areas. In certain rural areas up to 85 to 90% of the children were Schick-positive, and in one town the percentage varied with social conditions as observed elsewhere. For example, in Edinburgh, 11% of the school children of well-to-do classes were Schick-negative, whereas 66% of the poorer classes were Schick-negative.

From 1926 onwards, few cases of diphtheria occurred in Reykjavik. Dungal[11] found that between the ages of 8 and 14 years, 81.9% of 814 children gave a positive Schick reaction. Seven years later, Sigurjónsson[12] found that 89.6% of the 1603 school children examined in the same town were Schick-positive. Bergman and Melin[13] compared the Schick reaction in 1662 persons in Stockholm in 1930 and in 1942–43. The chances of infection and immunization were much greater in the earlier period. Whereas in 1930, 73% were Schick-negative, this figure had fallen to 46.6% in 1942–43. These investigations suggest that the diphtheria immunity of a community declines wherever the risk of latent infection is smallest.

As already pointed out, there was a slow and uniform decline in the diphtheria morbidity in Bergen until 1941. Not a single death from diphtheria was recorded in the period from 1935 to 1940. It should be noted that during this period diphtheria morbidity was also low elsewhere in Norway. Thus, in Oslo (about 270,000 inhabitants) only 4 cases of diphtheria were reported in 1938 and only 3 in 1939. In 1941 Strøm[14] applied the Schick test to 408 school children in Oslo. Of these, 98% were Schick-positive, 6 gave a negative and 2 a doubtful reaction. Only 25 adults were tested, 11 of them being doctors and 14 being technical assistants ranging in age from 21 to 55 years. Of these, 23 were positive and only 2 were negative. After Oslo also had undergone an epidemic of diphtheria, 232 unvaccinated children were tested in 1946, and 70% were found to be Schick-positive.[15]

Between 1937 and 1943 diphtheria was rare in Copenhagen. In 1943, a violent outbreak occurred in many

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adults as practically all the children had been vaccinated since 1941. Ipsen,[16] by means of Jensen's intracutaneous rabbit method, found no striking difference in the distributions of antitoxin titers in random groups of the population in 1937 and at the end of 1944. In 1937, 35.2% out of 289 persons over the age of 30 had an antitoxin titer under 0.01 units/ml, whereas in 1944, 45.6% of 261 showed this same antitoxin level. These observations show that even a year after a violent outbreak of diphtheria the natural diphtheria antitoxin content had not risen in the adults in Copenhagen.

In Bergen, the proportion of Schick-positive persons is larger and the antitoxin titer lower than elsewhere. Similarly, the antitoxin titers of the adult population appear to be lower. This finding is remarkable in view of the housing shortage due to war. This low antitoxin spectrum may have been the result of diphtheria vaccination.

Since diphtheria is mainly a disease of childhood, the number of adult patients over the age of 30 was negligible in earlier epidemics. Mass vaccination of school children and adolescents, however, has practically banished the disease from them and made it more prevalent in older age groups. Persons most exposed to infection are contacts near diphtheria patients or diphtheria carriers. Undetected and neglected cases of nasal diphtheria in children play a more important part than a few diphtheria carriers. At times, however, carriers are so numerous that they are of epidemiological importance. Should the number of such carriers rise in a small artificially immunized community, they might constitute an increased danger to the unimmunized. Thus, Dudley et al[8] have found that, in a boarding school, the number of carriers of virulent diphtheria strains increased among the vaccinated persons. On the other hand, Faragó[17] found that the immunization of at least 4 to 5 year groups did not increase the morbidity among the unvaccinated children. In Canada, where active immunization with diphtheria toxoid has been carried out on a large scale since 1926, Fitzgerald et al[18] have found that diphtheria has declined more than could have been expected from the number of persons immunized. Swabbings of the throats and noses of 3307 actively immunized school children from 7 different school districts in Bergen[19] failed to show any increase in the carrier rate (1.8%).

Such findings may be the result of the following: Children come into much closer contact with each other than adults. When diphtheria in childhood and adolescence disappears in response to vaccination and when the carrier rate is not increased in these groups, sources of infection become less numerous, and adults who are not actively immunized come thus indirectly to benefit from vaccination. Thus, in spite of the fact that the antitoxin spectrum of such a community remains low, the possibilities of spread of infection become small.

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SUMMARY AND CONCLUSIONS

During World War II and after a comparatively long period with a very low diphtheria morbidity, an epidemic of diphtheria broke out in Bergen with *typus gravis* gradually becoming the completely dominant type.

The antitoxin titer of 1000 persons directly after the termination of this epidemic was determined, and the antitoxin spectrum of the adult population of Bergen was found to be low. Fifty-nine percent of 716 unvaccinated persons had an antitoxin content of 0.01 units/ml or less, fifty-three percent over the age of 30. Eighty-eight unvaccinated persons who had previously suffered from diphtheria had similarly low antitoxin titers. Of 196 persons vaccinated with diphtheria toxoid, 93% had an antitoxin titer of more than 0.01 units/ml.

The extensive vaccination which has been carried out against diphtheria of late years in Bergen, notably among school children and young persons, seems to have had an influence on the antitoxin spectrum. The consequent reduction in the number of sources of infection indirectly benefits the unvaccinated adult members of the community. By reducing the spread of *Corynebacterium diphtheriae*, the antitoxin spectrum of the community remains low.

STREPTOMYCIN STUDIES IN TULAREMIA. I. THE EFFECT OF STREPTOTHRICIN AND STREPTOMYCIN ON *BACTERIUM TULARENSE* IN VITRO AND IN VIVO (MOUSE)

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Waksman and others[1–5] have shown that streptomycin and streptothricin have the desirable property of being bacteriostatic and bactericidal for a variety of gram negative organisms. Infections with *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella schottmuelleri*,[6] *Mycobacterium tuberculosis*,[7,8] *Brucella abortus*,[9] *Hemophilus pertussis*,[10] *Pasteurella pestis*,[11] *Bacterium tularense*[12] and other bacteria have been treated successfully in experimental animals.

Streptomycin has been found to be effective in man against infections with

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This work was done using the original streptomycin "S" unit described by Waksman.[2,3] All streptomycin concentrations reported in this series of papers have been converted to μg of pure streptomycin base in accordance with the recommendations of the Food and Drug Administration (quoted by Merck & Co.). The conversion factor used was 1 unit of streptomycin equals 1 μg (0.001 mg) of streptomycin base.

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Myco. tuberculosis,[13,14] *Bact. tularense*,[15,16] *Eberthella typhosa*,[17] and miscellaneous infections with a number of gram negative rods.

In this paper and in the two succeeding communications[18,19] of this series, we wish to present the results of experiments on the in vitro action of streptothricin and streptomycin[2] on *Bact. tularense* and the therapy of experimental tularemia infections in mice, rats and monkeys.

METHODS

The in vitro limits of bacteriostatic and bactericidal concentration of streptothricin and streptomycin were defined using varying numbers of *Bact. tularense* suspended in broth. The highly virulent Schu strain of *Bact. tularense* was used throughout because of the ease with which small numbers of this organism could be detected by mouse inoculation and because it could be cultivated in a liquid peptone medium.[20] In making the initial suspension of *Bact. tularense*, a 24 hour culture was scraped from the surface of a glucose cysteine blood agar (GCBA) slant and diluted in physiological saline to match a Fuller's earth standard of 500. Numerous colony counts[21] and mouse titrations showed that such a suspension contained 1 to 2 billion organisms per ml. The suspensions were diluted on this basis to contain the number of organisms indicated in the tables. For each experiment a count of the organisms in the original suspensions was made and, in addition, mouse titrations were usually done.

All dilutions other than the original suspension were made by transferring 1 ml of the suspension into 9 ml of the peptone medium devised by Snyder et al.[20] The antibiotic diluted in distilled water and Seitz-filtered was added so that the desired final concentration was obtained in μg per ml of streptomycin[3] or units per ml of streptothricin. The growth and survival of the organisms were measured by turbidity readings made after 8, 16 and 24 hours' incubation at 37 C and by quantitative plate counts. Because of the great susceptibility of the mouse to *Bact. tularense*, the bactericidal effect of the antibiotics was tested not only by plate counts but also by the intraperitoneal injection of 0.25 ml of undiluted and 10^{-3} and 10^{-6} dilutions of the culture into each of 6 mice. The mice were observed for 10 days before being discarded as uninfected. Any mice dying within this time were autopsied, and cultures were made from the heart blood and spleen.

In the in vivo therapeutic tests, 18 to 20 g white mice were inoculated intradermally with 15 to 20 MLD of fully virulent *Bact. tularense*. All streptomycin and streptothricin was administered subcutaneously. Controls received the same number of organisms but no drug. Drug controls for toxicity received the same amount of drug as the infected mice. The cause of death was established by autopsy of a suitable number of animals dying on each day. Most experiments were set up in duplicate; one set of animals was observed for survival and the other set was killed at intervals during and after treatment. Cultures were made from the heart blood, liver and spleen of these animals, and tissues were saved for histopathological examination. A detailed report of the pathology in treated and untreated animals will be made in a subsequent publication.

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IN VITRO STUDIES

Preliminary experiments on the bacteriostatic effect of streptothricin showed that with an original inoculum of 5 million bacteria per ml, a concentration in broth of 1 unit per ml inhibited the growth of *Bact. tularense*, and that 0.1 unit per ml failed to do so. With 10 units per ml the bacterial count was materially reduced after 30 minutes' exposure, and the culture was sterilized in 6 to 8 hours, whereas 24 hours was required to sterilize the culture with 1 unit per ml.

The results of several duplicate experiments using streptomycin are given in tables 1, 2 and 3. The broth tubes containing 0.001 to 10 μg of streptomycin per ml were seeded with *Bact. tularense* so that separate sets contained 1 million to 100 million organisms per ml. These tubes were incubated at 37 C and read grossly for turbidity at 8, 16 and 24 hours.

Turbidity readings showed that with an original inoculum of 1 million organisms, growth was inhibited by 0.01 μg per ml but not by 0.001 μg per ml. With 10 million and 100 million organisms per ml, growth was inhibited by 1 μg of streptomycin but not by 0.1 μg. After 24 hours plates made from tubes containing originally 1 million organisms and 0.1 μg of streptomycin were sterile. Plates made from tubes containing 10 million organisms and 1 μg of strepto-

TABLE 1.—*Bacteriostatic effect of streptomycin on* Bacterium tularense *in peptone broth.*

Inoculum—organisms per ml	Hours incubation	Amount of growth in presence of streptomycin concentration* of					
		0	0.001	0.01	0.1	1	10
10^6	0	−	−	−	−	−	−
	8	−	−	−	−	−	−
	16	+†	+	−	−	−	−
	24	2+	+	−	−	−	−
10^7	0	−	−	−	−	−	−
	8	3+	+	−	−	−	−
	16	5+	+	+	−	−	−
	24	5+	3+	2+	+	−	−
10^8	0	+	+	+	+	+	+
	8	5+	3+	+	+	−	−
	16	5+	3+	+	+	−	−
	24	6+	4+	2+	+	−	−

* Drug concentration expressed in μg per ml.
† Growth is indicated on scale of 1+ to 10+, in which 10+ matches in turbidity a T500 Fuller's earth standard.

TABLE 2.—*Bactericidal effect of streptomycin on* Bacterium tularense *in peptone broth.*

Inoculum—organisms per ml	Streptomycin (μg per ml)	Organisms per ml after 24 hours*
10^6	0	133×10^6
	0.001	107×10^6
	0.01	122×10^6
	0.1	0
	1.0	0
	10.0	0
10^7	0	173×10^6
	0.001	175×10^6
	0.01	174×10^6
	0.1	126×10^6
	1.0	0
	10.0	0
10^8	0	169×10^7
	0.001	183×10^7
	0.01	196×10^7
	0.1	137×10^7
	1.0	0
	10.0	0

* Colony counts were made in quadruplicate.

TABLE 3.—*Survival of mice injected with suspensions of* Bacterium tularense *which had been in contact with streptomycin for 24 hours.*

Drug concentration in μg per ml of broth	Inoculum of tubes before incubation organisms per ml		
	111×10^4	125×10^5	113×10^6
0	6/6*	6/6	6/6
0.001	6/6	6/6	6/6
0.01	6/6	6/6	6/6
0.1	0/6	6/6	6/6
1.0	0/6	0/6	4/6
10.0	0/6	0/6	0/6

* Mortality ratio. Numerator indicates the number of mice which died of tularemia infection, the denominator the number of mice inoculated.

mycin and 100 million and 10 μg, respectively, were also sterile.

The mouse titrations showed that broth containing 1 μg per ml with an original inoculum of 10 million organisms was sterilized; but with an inoculum of 100 million organisms, although the plates were sterile, 4 out of 5 mice injected succumbed to tularemia. The sterilizing concentration then, where 100 million organisms were used, was between 1 and 10 μg of streptomycin per ml.

TABLE 4.—*Time necessary to kill* Bacterium tularense *in broth containing varying dilutions of streptomycin.*

Concentration of drug in μg per ml of broth	Cultures sterile* after
10,000	Less than 30 minutes
1,000	30–60 minutes
100	60–120 minutes
10	6–8 hours
1	16–24 hours
0.1	Good growth in 24 hours
0.01	Good growth in 24 hours
0	Good growth in 24 hours

Inoculum: 5 million organisms per ml.
* 0.1 ml of undiluted broth and of logarithmic dilutions was plated on GCBA, and 0.25 ml of undiluted broth and dilutions of 10^{-3} and 10^{-6} were injected intra-abdominally into each of 5 mice.

Since these experiments showed a definite bactericidal as well as bacteriostatic effect, it seemed desirable to ascertain the length of time the drug must be in contact with the organisms in order to exert its bactericidal effect. Broth tubes seeded with 5 million organisms per ml and containing 0.1 to 10,000 μg of streptomycin per ml were sampled at intervals as indicated in table 4. The samples were tested for the presence of viable *Bact. tularense* by plating logarithmic dilutions on GCBA plates and by injecting mice intraperitoneally with 0.25 ml of undiluted samples and 0.25 ml of 10^{-3} and 10^{-6} dilutions.

All tubes containing 1.0 to 10,000 μg of streptomycin per ml were sterile in 24 hours. The greater concentrations of the drug killed all organisms in less than one-half hour. One-tenth μg and 0.01 μg per ml permitted growth of *Bact. tularense.*

In order to determine more precisely the lowest effective bacteriostatic and bactericidal concentrations of streptomycin, a series of broth tubes containing streptomycin in concentrations of 0.1 to 10 μg per ml were seeded with 5 million organisms per ml. The tubes were read for turbidity as an indication of growth at 6, 12, 18, 24, 48, and 72 hours. In 6 hours, only those tubes containing no drug or 0.1 μg per ml were slightly hazy with growth. At 12 hours the tubes containing 0.2 μg per ml were turbid. At 18 hours and thereafter tubes containing 0.4 to 10 μg per ml were clear, and all containing 0.2 μg or less were cloudy with growth. Samples from these tubes plated at once and after 24 and 48 hours determined the numbers of organisms actually present. The data are presented in table 5.

The quantitative data parallel the turbidity readings very well in that the dividing line between unhindered multi-

TABLE 5.—*Plate counts on* Bacterium tularense *in peptone broth containing streptomycin.*

Drug concentration μg per ml of broth	Number of bacteria present*		
	Time after addition of streptomycin		
	0–½ hr.	24 hr.	48 hr.
0	5,100,000	3,500,000,000	3,800,000,000
0.1	4,900,000	3,200,000,000	4,100,000,000
0.2	4,700,000	2,900,000,000	3,100,000,000
0.4	4,500,000	60,000	22,000
0.6	5,200,000	120	100
0.8	4,600,000	20	10
1.0	3,200,000	0	0
10.0	3,000,000	0	0

* All tubes were inoculated with 5 million organisms per ml.

plication and slight or no growth is 0.4 μg per ml. At 0.4 μg after 24 hours, 1/100 of the original number of *Bact. tularense* were living. After 48 hours this number had decreased, but viable organisms were still present. At 0.8 μg per ml a very small number remained after 24 hours, and at 1 μg per ml no organisms were viable.

Organisms that grew in the presence of 0.1 μg and 0.2 μg of streptomycin per ml were normal in size and appearance, but colonies from tubes containing 0.4 to 0.8 μg grew slowly on subculture and the stained smear showed a preponderance of very large forms. Enlarged coccoid, bacillary, oval, sickle-shaped and bizarre branching forms were seen. The percentage of the abnormal forms was greater from tubes containing 0.8 than from tubes containing 0.4 μg per ml. This unusual morphology did not persist beyond the second subculture on GCBA.

IN VIVO STUDIES

Two hundred units of streptothricin protected 88% of mice inoculated with 15 to 20 MLD of *Bact. tularense* when administered at the time of inoculation and every 24 hours for 10 days. Double this amount, or 500 units in a single dose, was lethal for mice. The use of streptothricin was abandoned because of its toxicity, and the work with streptomycin is presented below in more detail.

Preliminary therapeutic experiments in mice with crude streptomycin demonstrated that 50% survived a 20 MLD inoculum with virulent *Bact. tularense* if treated with 50 μg per day administered as a single subcutaneous dose for 8 days, starting at the time of infection. However, this dose was ineffective when therapy was delayed 24 hours after infection. All untreated infected controls died within 6 days, and the drug controls survived for 20 days.

Similar treatment with a single daily dose of 200 μg per day for 8 days protected 92% of the mice if therapy was instituted at the time of infection; while infected controls were all dead by the 5th day after inoculation, and all drug controls survived. Two hundred μg protected only 60% of the animals when administered in two or three divided doses every 8 or 12 hours. If therapy was delayed until 24 hours after inoculation, 200 μg given in either single or divided doses protected only 4 to 8% of the animals.

These preliminary tests appeared very promising and the following studies were carried out using better lots of streptomycin. The results presented in figure 1 demonstrate the effect of a single prophylactic dose of streptomycin given at the time of inoculation with 15 to 20 MLD. Four groups of 25 mice were treated with 50, 100, 200 or 500 μg of streptomycin. Two groups of drug control mice of 25 each were given 200 and 500 μg, respectively. Twenty-five mice were inoculated with the same number of organisms as the treated mice and constituted the untreated controls. The results showed that 200 to 500 μg of streptomycin administered at the time of inoculation permitted 92% survival, whereas a single prophylactic dose of 50 to 100 μg was relatively ineffective. The untreated controls all succumbed and only one drug control animal died.

It is obvious that in the treatment of tularemia, streptomycin, to be useful, must be effective after the onset of symptoms. Since early experiments indicated that the time at which therapy was initiated in mice determined the effectiveness of treatment, tests were next carried out to determine whether mice would recover if therapy were delayed until after the infection had become well established. Accordingly, treatment was delayed until 48 hours after inoculation, since it was demonstrated that mice

inoculated with 15 to 20 MLD developed focal liver lesions within this time. In the experiments illustrated in figure 2, 6 groups of 30 mice each were given 50, 100, 150 and 200 μg of streptomycin every 3 hours for a 10-day period starting 48 hours after infection. Infected untreated controls and uninfected drug-treated controls were included as indicated. It will be seen from figure 2 that 200 μg of streptomycin given every 3 hours for 10 days was the minimal effective dose sufficient to protect 96% of the mice; treatment with 150, 100 and 50 μg every 3 hours was progressively less effective.

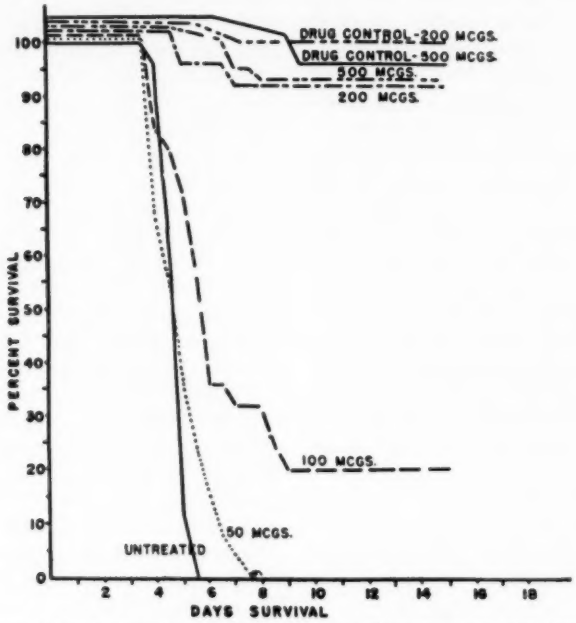

FIG. 1.—Survival of mice after single subcutaneous doses of streptomycin administered at the time of infection. Mice were inoculated intradermally with 15 to 20 MLD.

In a series of animals similarly infected and treated but killed at intervals subsequent to therapy, the spleens were emulsified and inoculated into 6 normal mice. It was evident from the specific deaths in these mice that, although 150 to 100 μg therapy permitted 75 and 55% survival, respectively, 20 to 60% of these animals harbored the organisms in the spleen as late as 50 days after challenge. On the other hand, only an occasional animal treated with 200 μg harbored *Bact. tularense* in the spleen 30 days after inoculation. This experiment showed not only that 200 μg was the minimal effective dose when treatment was started 48 hours after infection, but also that if the drug dosage was not sufficient, there was danger of a persistent latent infection in the surviving animals.

Having established the minimal effective dose against 20 MLD as 200 μg administered every 3 hours for 10 days, it seemed advisable to determine how late

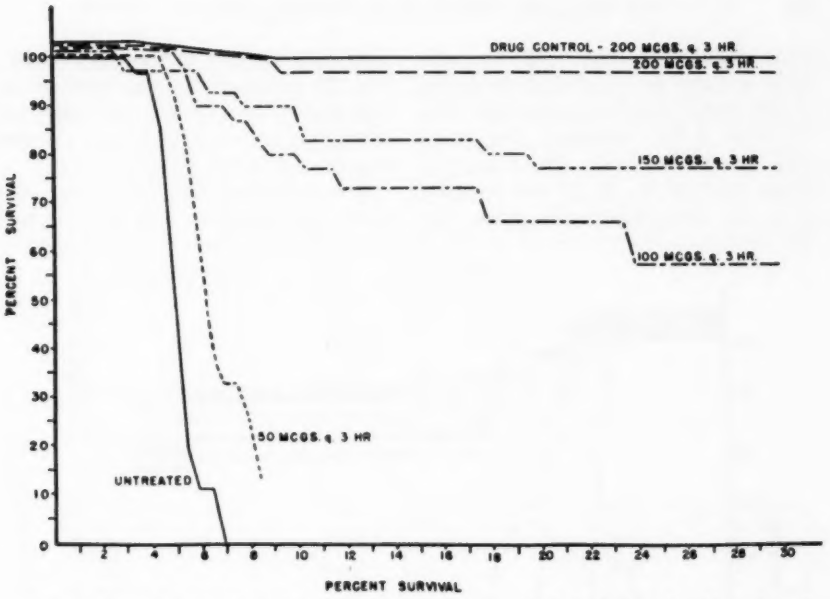
DRUG CONTROL - 200 MCGS. q 3 HR.
200 MCGS. q 3 HR.
150 MCGS. q 3 HR.
100 MCGS. q 3 HR.
50 MCGS. q 3 HR.
UNTREATED
PERCENT SURVIVAL
PERCENT SURVIVAL

FIG. 2.—Effect of repeated small doses of streptomycin on survival of mice inoculated intradermally with 15 to 20 MLD. Streptomycin therapy was begun 48 hours after infection and administered every 3 hours for 10 days.

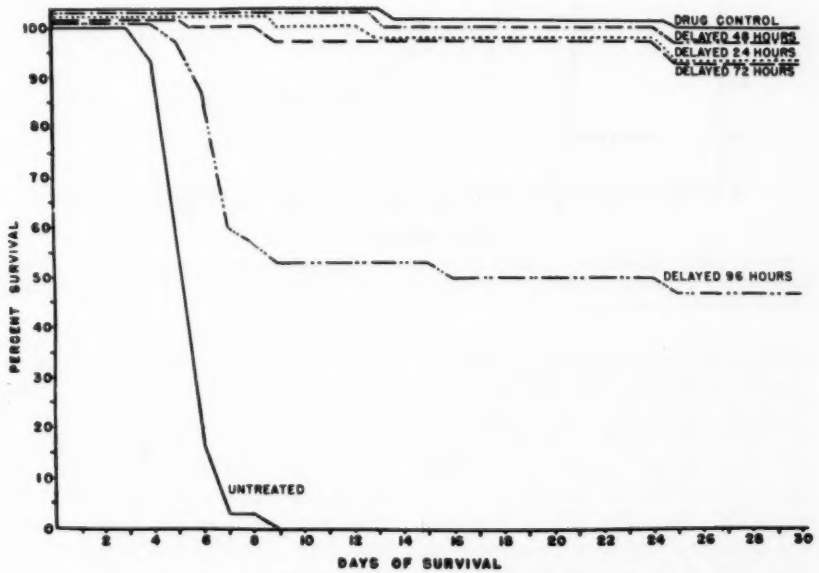

FIG. 3.—Effect of delayed therapy on survival of mice inoculated intradermally with 15 to 20 MLD. Therapy was initiated 24, 48, 72 and 96 hours after infection. Dosage was 200 μg per 20 g mouse administered subcutaneously every 3 hours for 10 days.

after infection therapy could be instituted and still be effective. Five groups of 100 animals each were injected with 20 MLD of *Bact. tularense*. One group each was treated with 200 μg every 3 hours, starting 24, 48, 72 and 96 hours after infection, and the 5th group was left untreated. An additional group of 100 animals was not infected but was treated for 10 days with 200 μg every 3 hours. It can be seen from figure 3 that the treatment resulted in survival of over 90% of the animals, even when therapy was delayed for as long as 72 hours after infection. When treatment was delayed until 96 hours after infection, 46% of the animals survived. The survival of this number is particularly significant since at 96 hours after infection the untreated animals were weak, dehydrated and showed extensive necrotic skin lesions, together with severe hepatic and splenic damage.

The following experiment was designed to determine how long it was necessary to continue treatment (200 μg every 3 hours) to obtain maximal recovery and to eliminate latent infections. The experiment was set up in duplicate; in the first group, surviving mice were killed to ascertain the percentage of animals showing latent infection, and in the second group, the animals were observed to obtain statistical survival rates.

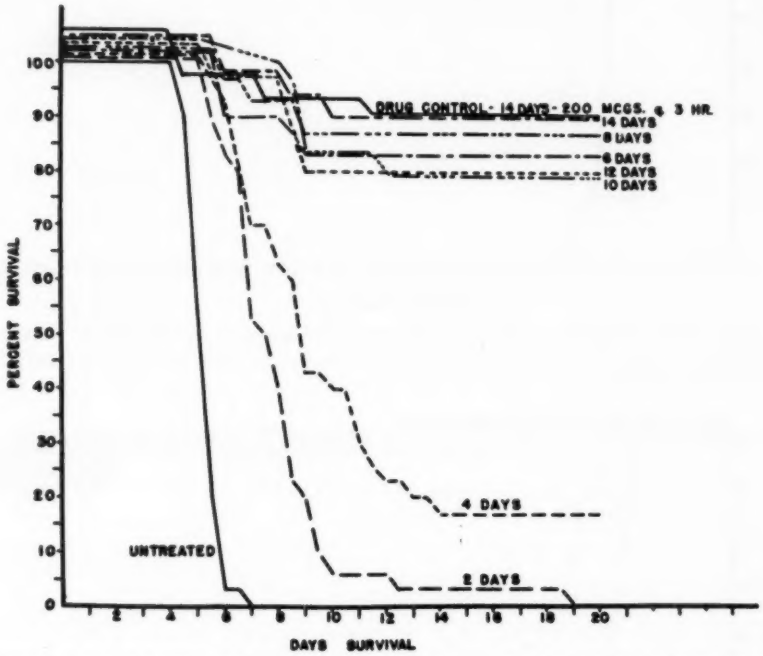

FIG. 4.—Effect of duration of therapy on survival of mice inoculated with 15 to 20 MLD. Therapy was initiated 48 hours after infection. Dosage was 200 μg per 20 g mouse administered subcutaneously every 3 hours for 2 to 14 days.

Figure 4 shows the results of 2, 4, 6,

8, 10, 12 and 14 day periods of treatment when 200 μg were administered every 3 hours to 30 mice in each group starting 48 hours after infection with 20 MLD. It is evident from the figures that treatment at this level for 2 or 4 days, although it prolonged life, saved relatively few of the animals. Treatment for 6 to 14 days was required to save 80% of the animals. Cultures of tissues on GCBA medium indicated that during streptomycin therapy *Bact. tularense* was first eliminated from the blood stream, then from the liver and subsequently from the spleen. With 6 days of therapy, 80% of the survivors harbored *Bact. tularense* in the spleen as late as 50 days after inoculation (fig. 5). Spleens from animals treated for 10 and 14 days did not contain *Bact. tularense* 30 days after inoculation. Eight-day therapy produced intermediate results. This experiment demonstrated that it was necessary to continue low dosage therapy (200 μg every 3 hours) for at least 8 days, and preferably for 10 to 14 days, to obtain good survival and to avoid latent infections in the survivors. The results of the experiment are shown in figure 5.

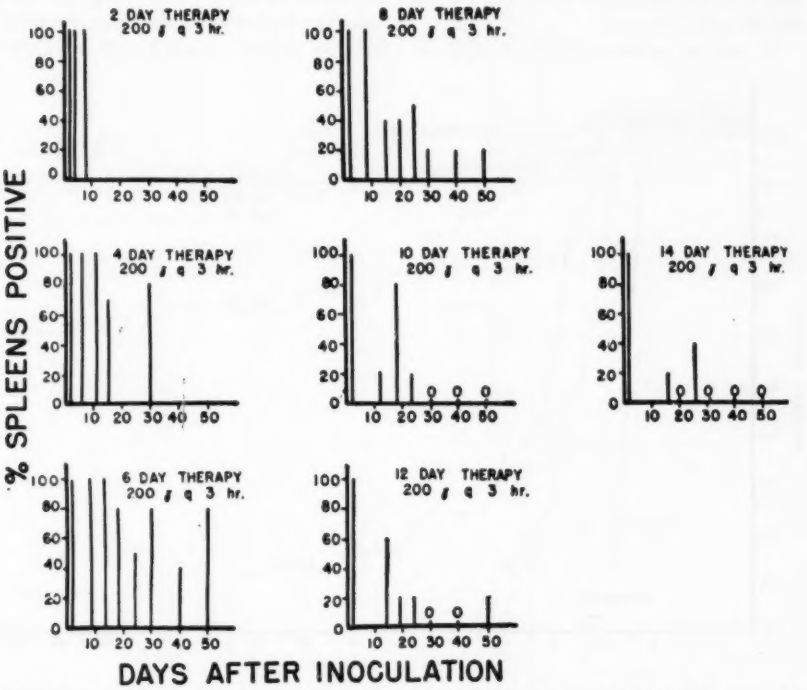
2 DAY THERAPY 200 γ q 3 hr.
8 DAY THERAPY 200 γ q 3 hr.
4 DAY THERAPY 200 γ q 3 hr.
10 DAY THERAPY 200 γ q 3 hr.
14 DAY THERAPY 200 γ q 3 hr.
6 DAY THERAPY 200 γ q 3 hr.
12 DAY THERAPY 200 γ q 3 hr.
% SPLEENS POSITIVE
DAYS AFTER INOCULATION

FIG. 5.—Recovery of *Bacterium tularense* from the spleen of mice treated with 200 μg every 3 hours for 2 to 14 days.

The effectiveness of a larger dosage of streptomycin over a short period of treatment was next investigated. This experiment was also done in duplicate, one lot of mice being sacrificed to ascertain the presence of latent infection and

the possible toxic effects at this dosage, and the other lot to obtain statistical survival rates.

In each lot, groups of 25 mice were treated with 1000 μg of streptomycin every 3 hours. Treatment was started 48 hours after inoculation with 20 MLD and was discontinued after 12, 24, 36, 48, 60 and 72 hours.

It will be seen in figure 6 that although all of the mice treated for 12 hours succumbed, death was delayed for two or three days. There was a striking difference in the percentage survival in mice treated for 36 hours, since 60% survived as compared to those treated for 24 hours, in which less than 10% survived. Treatment continued for 60 to 72 hours gave 88 to 96% survival, whereas treatment for 48 hours resulted in 80% survival.

From these experiments it may be concluded that high dosage therapy over a short period of time is as effective in preventing death in infected mice as low dosage therapy over a long period.

While a dosage of 1000 μg every 3 hours for a 72 hour period was as effective in the reduction of mortality as 200 μg given for 10 to 14 days (fig. 4), it was apparently less effective than the smaller dose in preventing latent infections; the spleens of 50% of the mice treated for 72 hours and killed 50 days after inoculation still retained the organism. Although there was a gradual reduction in the percentage of latent infections following therapy, the rate appeared to be slower than in animals treated with 200 μg for 8 to 14 days (fig. 7).

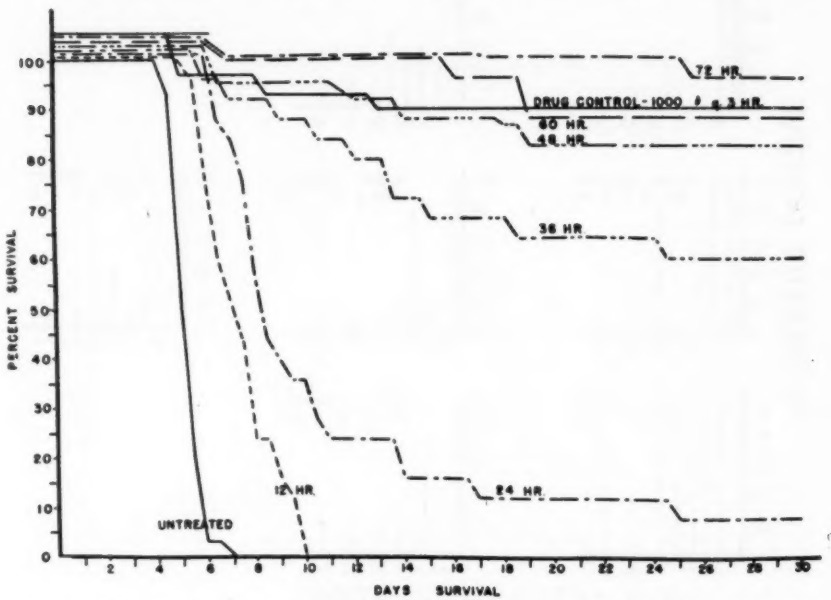

FIG. 6.—Effect of duration of 1000 μg therapy on survival of mice inoculated with 15 to 20 MLD. Therapy was initiated 48 hours after infection. Streptomycin was administered subcutaneously in a dose of 1000 μg per 20 g mouse every 3 hours and continued for 12 to 72 hours.

The subcutaneous LD_{50} of this lot of streptomycin was 16 mg per 20 g mouse or 16 times the dosage used in this experiment. However, cumulative toxic

effects may have developed over the 3-day period, since 8 deaths occurred in the drug controls treated with 1000 μg every 3 hours for 72 hours.

served at the end of the treatment period. Mild fatty infiltration of the liver cells persisted for 17 days after therapy was discontinued. Throughout the en-

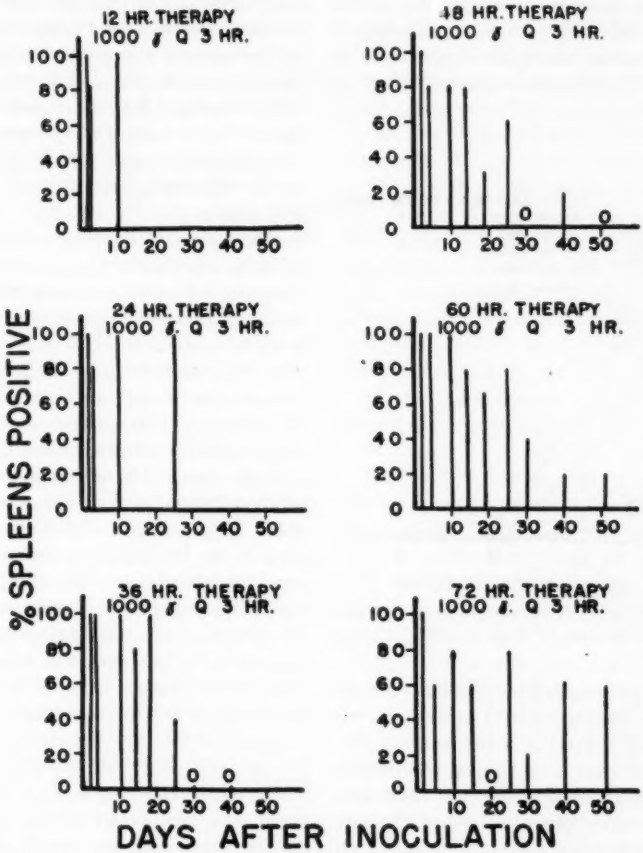

FIG. 7.—Recovery of *Bacterium tularense* from the spleen of mice treated with 1000 μg every 3 hours for periods from 12 to 72 hours.

Uninoculated drug control animals treated for 72 hours and killed for histopathological examination at the end of therapy, and at 10 days and 17 days after therapy had been stopped, showed the following changes, presumably caused by administration of the drug. Cloudy swelling of the liver was obtire period spleen sections showed mild generalized lymphoid hyperplasia. These pathological changes were not severe, but it is possible that the observed deaths in the drug control animals were in part due to delayed toxicity.

In all the above experiments there

was close correlation between the survival of treated animals, type and amount of treatment, recovery of organisms from the spleen and the pathological lesions observed in the spleen. When adequate therapy was initiated at the time of infection, no histopathological changes were observed in the liver or spleen. If treatment was delayed 48

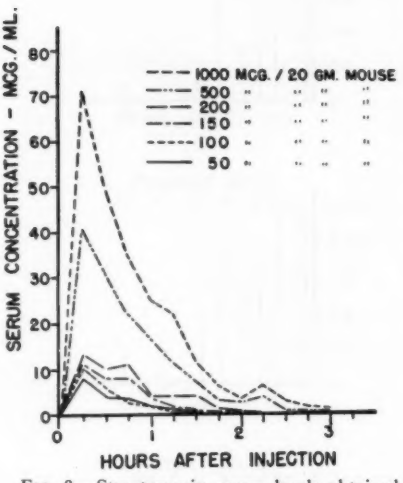

FIG. 8.—Streptomycin serum levels obtained after administration of single doses of the drug.

hours after inoculation, tissue damage did not progress after therapy was started. However, if treatment was discontinued after 2 or 4 days, the disease recurred and progressed to a fatal outcome. In mice given adequate therapy only healing focal lesions were observed during convalescence. A detailed report on the modification by therapy of the pathologic changes usually observed will be published later.

Resistance to streptomycin.—It is of interest to note that several observers have reported the occurrence of streptomycin-resistant strains of various bacteria, and while resistance to streptomycin can be induced in *Bact. tularense*,[22] none of the 75 strains recovered during these experiments had acquired such resistance. Strains were recovered from dead or killed mice from 4 to 50 days after inoculation, both during and after cessation of therapy. The mice had received therapy for 12 hours to 10 days at the various dosage levels described in this paper. All recovered cultures were fully virulent for mice and were inhibited by 0.4 μg of streptomycin when the antibiotic was added to peptone broth cultures as described previously in this paper.

Challenge of surviving mice.—To determine whether surviving animals were immune following recovery after streptomycin therapy, 3 groups of recovered mice were challenged 20 to 30 days after the original infection. A group of 40 mice had previously received one dose of 50 to 200 μg of streptomycin at the time of inoculation, another group of 90 mice, a single dose of 100 to 500 μg at the time of inoculation, and a third group of 51 mice, 200 μg daily for 10 days in a single dose or in divided doses starting at the time of inoculation. All of these mice succumbed when challenged with 10 to 20 MLD. An additional group of 50 mice which had survived when treatment was initiated from 48 to 96 hours after infection was reinoculated with 10 to 20 MLD 50 days after the original infection; only one animal survived.

Streptomycin serum levels.—An experiment was performed to determine the serum streptomycin levels obtained after various dosages of the drug. Pooled heart blood from 3 to 5 mice was obtained at 15 minute intervals for 3 hours following subcutaneous administration of streptomycin. The pooled serum was assayed immediately using the Stebbins-Robinson method.[23] The

22. Foshay, L. 1945, Personal communication.
23. Stebbins, R. B. and Robinson, H. J. 1945, A

results are presented in figure 8 and show that administration of 1,000 and 500 μg per 20 g mouse yielded peak serum levels of 72 and 40 μg per ml, respectively, with an appreciable blood level 2.5 hours after administration. A dose of 200 μg gave a maximal serum level of 13 μg per ml with an appreciable level for 2 hours. Administration of lesser quantities yielded progressively lower serum levels with no appreciable levels beyond 1.5 hours after administration. These streptomycin levels in serum are slightly higher than the whole blood levels reported by Forgacs and Kornegay[24] and Kornegay et al[25] using a different assay method.

DISCUSSION

The results of the in vitro tests indicate that *Bact. tularense* is very sensitive to streptothricin and streptomycin. Heilman[12] reported the inhibition of two stock strains of *Bact. tularense* on cystine blood agar containing 0.3 unit per ml of streptomycin. A virulent strain was inhibited by 0.15 unit per ml. Although the method of testing differed from ours, the results are comparable. Other gram negative organisms showing a comparable sensitivity in vitro are: *Br. abortus*,[9] *H. pertussis*,[10] *H. influenzae*,[26] *Past. pestis*,[11] *Eb. typhosa*,[27] *Escherichia coli*,[27] and some strains of *Klebsiella pneumoniae*.[28] Other bacteria appear to require larger amounts of streptomycin to inhibit growth or to give bactericidal effects when tested in vitro.

Our results on the chemotherapeutic effect of streptomycin in mice inoculated with *Bact. tularense* are comparable with those of Heilman[12] in spite of differences in the details of drug administration. In one group of 15 mice he obtained 100% survival when 1000 units were given each day in 5 doses for 10 days. Therapy was started 7 hours after infection. Our results show a survival of 96% in mice given a single dose of 200 μg at the time of infection. Heilman's results with 500 units daily for 10 days, which gave survival of 60% of the mice, are comparable to our preliminary results using 200 μg per day starting at the time of infection.

Heilman failed to isolate *Bact. tularense* from the spleens of 6 recovered mice killed 27 days after inoculation, but in another experiment he reports the isolation of *Bact. tularense* from the spleen of a mouse dying of pneumonia 26 days after inoculation. Our results on a larger number of mice indicated that a high percentage of mice harbored *Bact. tularense* in the spleen at the end of the therapy period under all schedules of treatment.

When recovered mice were challenged with 1000 MLD 28 days after the original inoculation, Heilman found that 1 out of 9 survived. When 100 MLD were used 23 days after inoculation, 6 out of 9 mice survived. In our experiments,

method for the determination of streptomycin in body fluids. Proc. Soc. Exper. Biol. & Med. **59**: 255–257.

24. Forgacs, J. and Kornegay, G. B. 1946, Studies on streptomycin. I. Assays in body fluids. J. Lab. & Clin. Med. **31**: 514–522.
25. Kornegay, G. B., Forgacs, J. and Henley, T. F. 1946, Studies on streptomycin. II. Blood levels and urinary excretion in man and animals. J. Lab. & Clin. Med. **31**: 523–534.
26. Durant, T. M., Sokalchuck, A. J., Norris, C. M. and Brown, C. L. 1946 Streptomycin therapy in *Hemophilus influenzae* pulmonary infection. J.A.M.A. **131**: 194–196.
27. Robinson, H. J., Smith, D. G. and Graessle, O. E. 1944, Chemotherapeutic properties of streptomycin. Proc. Soc. Exper. Biol. & Med. **57**: 226–231.
28. Heilman, F. R. 1945, Streptomycin in the treatment of experimental infections with microorganisms of the Friedlander group (Klebsiella). Proc. Staff Meetings, Mayo Clinic **20**: 33–39.

several groups of 40 to 90 treated mice were challenged with 10 to 20 MLD, 20 to 50 days after the first inoculation, and did not show any significant degree of immunity. These results do not agree with those of Heilman nor with our results on the challenge of vaccinated mice which had recovered from a previous sublethal infection.[29] In our experience, the normal mouse always succumbs to an infecting dose of *Bact. tularense*. In all groups of streptomycin-treated mice, 40 to 100% harbored organisms after therapy was discontinued. The fact that the mice were able to localize and gradually eliminate these organisms would indicate the existence of a certain degree of immunity. We are unable to explain, however, why such animals failed to resist challenge with minimal doses of *Bact. tularense*. Infected white rats treated with streptomycin have been found to develop a high degree of immunity,[18] and Foshay[15] has observed that human cases treated with streptomycin develop serum agglutinins to the same degree as recovered, untreated cases.

The successful treatment of tularemia in white mice with an antibiotic is notable in that this animal succumbs regularly to the administration of 1 to 5 organisms and at autopsy the organisms are numerous in the blood and tissues. So far as we know, streptomycin is the only therapeutic agent to be used successfully against experimental tularemia infections in mice. Streptothricin was also effective, but the lethal dose of the drug samples we have tested was only about twice the therapeutic dose, whereas the lethal dose of streptomycin was 50 to 80 times the therapeutic dose.

The minimum effective dose of streptomycin against an established infection in mice was 200 μg per 20 g mouse or 10,000 μg per kg of body weight. The dosage reported by Foshay[15] and the dosage on our reported human cases[17] was 500 μg per kg and 1666 μg per kg, respectively. The larger dosage required in the mouse is probably due to the greater susceptibility and lack of natural immunity in this animal.

29. Coriell, L. L., Downs, C. M. and Clapp, M. 1949, Studies on tularemia. Immunization of white mice. J. Immunol. In press.

SUMMARY

1. Streptothricin in a concentration of 1 unit per ml was bacteriostatic and bactericidal for *Bacterium tularense* in vitro.

2. Streptomycin was bacteriostatic in vitro in a concentration of 0.4 μg per ml and was bactericidal at 1 μg per ml.

3. The minimal effective dose of streptomycin necessary to protect 90% of mice against 15 to 20 MLD was 200 μg given every 3 hours over a period of 10 days when treatment was started 48 hours after infection. This dosage was still effective when treatment was delayed until 72 hours after infection.

4. A large percentage of the treated mice harbored organisms in the spleen for as long as 50 days unless treatment was continued for 10 to 14 days.

5. No streptomycin-resistant strains were developed during therapy.

6. The treated and recovered mice were not immune as determined by challenge inoculations.

STREPTOMYCIN STUDIES IN TULAREMIA. II. STREPTOMYCIN THERAPY IN WHITE RATS

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It has been shown[1] that streptomycin is highly effective in the treatment of experimental tularemia in the white mouse. These experiments gave convincing evidence of the efficacy of the drug since the white mouse has little or no resistance to tularemia,[2] and to be effective in this animal a therapeutic agent must eliminate the infection without much assistance from the defensive mechanisms of the host. As shown in paper I of this series,[1] mice which recovered under streptomycin therapy were not immune to reinfection.

Foshay[3] has reported on the successful use of streptomycin in human tularemia and has shown that the rise of serum agglutinins during convalescence is not interfered with by streptomycin therapy.

The white rat has been shown to have a considerable natural immunity to tularemia and in contrast to the mouse is easily immunized by vaccines and develops a solid immunity following a sublethal infection.[4] In these characteristics the white rat more nearly resembles man than does the mouse.

It was considered desirable, therefore, to ascertain whether treatment with streptomycin interferes with the development of immunity in an animal such as the rat, whose natural resistance approaches that of man. Accordingly, in the following experiments, groups of white rats were infected with *Bacterium tularense*, treated with streptomycin and the survivors were subsequently reinoculated.

METHODS

Streptomycin was dissolved in sterile saline and adjusted to pH 6.8 to 6.9 with 0.1 *N* sodium hydroxide. For both experiments described below, the concentration of streptomycin was accurately adjusted so that the required dose was administered subcutaneously in 0.5 ml. Because the available supply was limited, the drug was administered in one or two daily doses, rather than every three hours as in previous work.[1,5] Preliminary experiments established that this dosage schedule was adequate if therapy was initiated at the time of infection.

Adult rats (Tumblebrook strain) weighing approximately 200 g were used throughout this study. Animals were inoculated subcutaneously with 1 ml of a 10^{-1} dilution of a standard suspension of *Bact. tularense*, strain Schu. The standard suspension was prepared by emulsifying the growth from a 24-hour glucose cysteine blood agar (GCBA)[6] culture in physiological saline and diluting to match a T500 Fuller's earth standard. The test inoculum contained approximately 1000

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1. Chapman, S. S., Downs, C. M., Coriell, L. L. and Kowal, S. F. 1949, Streptomycin studies in tularemia. I. The in vitro effect of streptothricin and streptomycin on *Bacterium tularense* with special reference to streptomycin therapy in white mice. J. Infect. Dis. **85**: 25–38.
2. Downs, C. M., Coriell, L. L., Pinchot, G. B., Maumanee, E., Klauber, A., Chapman, S. S. and Owen, B. J. Studies on tularemia. I. Comparative susceptibility of various laboratory animals to *Bacterium tularense*. J. Immunol. In press.
3. Foshay, L. and Pasternak, A. B. 1946, Streptomycin treatment of tularemia. J.A.M.A. **120**: 393–398.
4. Downs, C. M., Coriell, L. L., Pinchot, G. B., Eigelsbach, H. and Owen, B. J. Immunization against tularemia in experimental animals. II. Immunization of white rats. J. Immunol. In press.
5. Chapman, S. S., Coriell, L. L., and Nelson, W. E. 1949, Streptomycin studies in tularemia. III. Streptomycin therapy in monkeys. J. Infect. Dis. **85**: 45–61.
6. Downs, C. M., Coriell, L. L., Chapman, S. S. and Klauber, A. 1947, The cultivation of *Bacterium tularense* in embryonated eggs. J. Bact. **53**: 89–100.

LD_{50} as determined by the Reed and Muench method[7] in preliminary titrations. The same dose was used for both the initial and the challenge inoculations.

In order to determine the cause of death and the course of the infection during therapy, heart blood, liver and spleen of animals which died or were killed were cultured by streaking on GCBA. Tissue for pathological study was fixed in Zenker's fluid and the cut sections stained with Giemsa, and hematoxylin and eosin.

EXPERIMENTAL

Experiment 1.—This experiment was designed to determine the effect of streptomycin on the course of the disease when therapy was begun early. Eighty rats were divided into 4 groups of 20 each. One group, which was not inoculated, served as a drug control; each rat in this group was treated with 2000 μg of streptomycin in a single daily dose for 10 days. Three groups were inoculated with the standard test dose of *Bact. tularense*, described under methods. One group of inoculated control animals was untreated. The other two groups were treated for 10 days with single daily doses of 2000 μg of streptomycin per rat, starting at the time of infection in one group and 24 hours after infection in the other group. Throughout the 10-day period of therapy and for 25 days thereafter the animals were examined every 6 hours and deaths were recorded. On the 35th day the surviving rats were given a challenge inoculum and were observed for an additional 20 days.

The results obtained in this experiment are shown in table 1. With the infecting dose used, 30% of the untreated animals survived. When therapy was initiated at the time of infection or 24 hours after infection, 90% of the animals survived. There were no deaths in the drug control group.

TABLE 1.—*Effect of streptomycin therapy on tularemia in white rats (experiment 1).*

Treatment initiated	Dosage in μg*	Therapy: Results: Number of animals alive on post inoculation day													Therapy: Results: Percent survival	Results of challenge inoculation†: Mortality ratio	Results of challenge inoculation†: Percent survival
		0	1	2	3	4	5	6	7	8	9	10	20	35			
Untreated	0	20	20	18	10	8	8	7	7	6	6	6	6	6	30	0/6	100
At time of infection	2000	20	20	20	18	18	18	18	18	18	18	18	18	18	90	3/18‡	83.3
24 hr. after infection	2000	20	20	19	19	18	18	18	18	18	18	18	18	18	90	1/18	94.4
Drug control	2000	20	20	20	20	20	20	20	20	20	20	20	20	20	100	13/20	35

* Given as μg of drug per 200 g body weight administered as a single daily dose for 10 days.
† Challenge dose 35 days after original infection.
‡ Two animals died of non-specific pneumonia.

Within 24 hours after inoculation the untreated rats appeared sick; they were listless and their coats were rough. Fifty percent of the rats were dead on the third day. The survivors were sick throughout the first 6 days of the disease, after which slow improvement in their condition was evident. By the 15th day after infection they appeared to be well.

Animals in which therapy was started at the time of infection showed only slight signs of illness 24 hours later and appeared normal by the 5th day of therapy; only 2 animals died. Rats in which therapy was delayed until 24

7. Reed, L. J. and Muench, H. A. 1938, A simple method for estimating 50 percent endpoints. Am. J. Hyg. **27**: 493–497.

hours after infection showed typical signs of illness before treatment was commenced. Two rats in this group died, but the remaining animals were noticeably improved by the third day of therapy. Recovery progressed in this treated group so that by the 10th day of therapy there were no obvious external signs of illness.

Survivors re-inoculated 35 days after the initial infection were almost completely immune (table 1). Two rats a control for drug toxicity. One group was inoculated as described in experiment 1 and received no treatment. The three remaining groups were inoculated, and the initiation of therapy was delayed until 24, 36 and 48 hours after inoculation, respectively. Blood, liver and spleen were cultured on GCBA, and blocks of liver and spleen were prepared for histologic examination.

Therapy was continued for 10 days, and observations were made every 6

TABLE 2.—*Effect of streptomycin therapy on tularemia in white rats (experiment 2).*

Treatment initiated	Dosage ing μg*	Therapy: Results: Number of animals surviving on post inoculation day													Therapy: Results: Percent survival	Results of challenge inoculation†: Mortality ratio	Results of challenge inoculation†: Percent survival
		0	1	2	3	4	5	6	7	8	9	10	15	20			
Untreated	0	32	23	30	19	12	8	7	6	6	6	6	4	4	12.8	0/4	100
24 hrs. after infection	2000	32	32	30	29	28	28	28	28	27	27	27	27	27	84.4	1/27	96.3
36 hrs. after infection	2000	32	32	31	24	22	21	20	19	19	19	18	18	18	56.2	0/18	100
48 hrs. after infection	2000	32	32	32	13	10	9	9	8	8	8	8	8	8	25.0	0/8	100
Drug control	2000	32	32	32	32	32	32	32	32	32	32	32	32	32	100	21/32	34.4

* Given as μg of drug per 200 g body weight administered as a single daily dose for 10 days.
† Challenge dose 20 days after the original infection.

which had recovered from the first infection succumbed to pneumonia after the challenge inoculation, but since *Bact. tularense* could not be recovered from them at autopsy, their deaths must be attributed to causes other than tularemia.

Experiment 2.—Experiment 1 demonstrated that streptomycin was effective if therapy was started at the time of infection or 24 hours after infection. A second experiment was designed to see whether the drug would be effective if treatment were delayed longer than 24 hours. In this experiment 2000 μg of streptomycin per 200 g of body weight were administered twice daily. One hundred and sixty rats were divided into 5 equal groups. One group of animals received streptomycin only and served as hours for 20 days. Animals alive on the 20th day were tested for immunity by the inoculation of 1000 LD_{50} of *Bact. tularense* as described in experiment 1.

The effect of treatment on survival and the results of challenge in treated animals after recovery are shown in table 2. Twelve and one-half percent of the untreated rats survived for 20 days after infection. In the treated groups, when therapy was instituted 24 hours after infection, 84% of the animals survived. If therapy was delayed for 36 or 48 hours, 56 and 25%, respectively, of the animals survived.

As in experiment 1, the untreated rats were obviously ill within 24 hours after inoculation. They became progressively worse and were prostrate by 48 hours. Thirty-seven percent of the untreated

TABLE 3.—*Effect of streptomycin therapy on persistence of* Bacterium tularense *in infected rats (experiment 2).*

Treatment initiated	Presence of *Bacterium tularense* in blood, liver or spleen of rats examined post-inoculation at											
	24 hours		36 hours		48 hours		7 days		12 days		20 days	
	No. of rats examined	Results	No. of rats examined	Results	No. of rats examined	Results	No. of rats examined	Results	No. of rats examined	Results	No. of rats examined	Results
Untreated	5	B 5+ L 5+ S 5+					3	B 3+ L 3+ S 3+	3	B 1+2− L 1+2− S 3+	3	B 1+2− L 2+1− S 3+
24 hr. after infection	5	B 5+ L 5+ S 5+					3	B 3− L 1+2− S 3+	3	B 3+ L 3− S 3−	3	B 3+ L 3− S 3−
36 hr. after infection			5	B 5++ L 5++ S 5++			3	B 3− L 3++ S 3++	3	B 3− L 3− S 3−	3	B 3− L 3− S 3−
48 hr. after infection					5	B 5++ L 5++ S 5++	3	B 3− L 3++ S 3++	3	B 3− L 3− S 3−	3	B 3− L 3− S 3−

Tissues were cultured on glucose-cysteine-blood agar. B—blood, L—liver, S—spleen. Streptomycin dosage—2000 μg twice daily for 10 days.

rats had died by the 4th day. Seven days after inoculation the surviving untreated rats were improved, began to gain weight and were fully recovered by the 15th post-inoculation day.

In all series, those rats which were treated showed an obvious improvement in their condition within 24 hours after therapy was initiated. The animals were active, ate well and appeared to have recovered, except for a slightly roughened coat, by the 7th day after infection, and had completely recovered by the 10th day.

Fifty-seven animals surviving from all infected groups in experiment 2 were challenged 20 days after the initial inoculation. The animals used as normal controls had been used as drug controls but had received no organisms. Ninety-eight percent of the recovered animals survived the challenge dose, while 34.4% of the normal animals succumbed. This appeared to be a clear indication that streptomycin therapy did not interfere with the development of immunity in infected animals.

The results on the cultures made from heart blood, liver and spleen before, during and after therapy are summarized in table 3. These showed that the *Bact. tularense* was cultured from the blood, liver and spleen of all untreated animals within 24 hours after inoculation. Untreated rats killed on the 7th day of infection gave positive blood, liver and spleen cultures, but of those rats killed 12 days after infection, only one of three showed positive blood and liver culture although all harbored the organism in the spleen. In the rats killed 20 days after infection one of three showed positive blood culture, two of three positive liver culture and all showed positive spleen culture.

In the treated animals, the blood stream was free of *Bact. tularense* by the 7th day, but the organism was still

present in the spleen of all, and in the liver of 7 out of 9 sampled. When therapy was started 24 hours after infection, only one of three animals showed the organism in the liver at 7 days; but when therapy was initiated later, the organism was cultured from the liver of all rats at 7 days. In contrast to the untreated animals, no positive cultures were obtained from the blood, liver or spleen of treated animals when they were examined 12 and 20 days after infection.

Pathology

A detailed report of the modification produced in the pathologic picture of tularemia in various laboratory animals by treatment with streptomycin will be published later. Only a few findings are presented here to illustrate the degree of tissue damage at the time therapy was started and to compare the treated and untreated animals examined by necropsy at the end of therapy.

Untreated animals killed 24, 36 and 48 hours after subcutaneous inoculation showed, in varying degree, the same gross lesions, namely, subcutaneous congestion, enlarged and congested but not necrotic regional lymph nodes, an enlarged dark red spleen and a normal to swollen, congested liver. Foci of necrosis in the spleen or liver were not observed by gross examination.

Microscopic examination of liver sections from animals killed 24 hours after infection showed swollen liver cords with occasional small areas of fibrin deposition infiltrated with polymorphonuclear and an occasional mononuclear leucocyte. The liver cells in these areas were swollen and granular. In some animals killed 36 hours after infection, fatty infiltration of liver cells was observed, and the areas of focal hepatitis consisted of groups of polymorphonuclear leucocytes surrounding necrotic liver cells. Forty-eight hours after infection, the areas of focal hepatitis were more numerous, and in some, foci groups of 10 to 20 liver cells were necrotic or stained poorly.

Spleen sections from animals killed 24 hours after infection showed mild lymphoid hyperplasia, and small areas in the pulp contained polymorphonuclear leucocytes. At 36 and 48 hours after infection the sections from 10 animals showed a range from moderate congestion to coagulation necrosis of large areas of the red pulp. Karyorrhexis was prominent in the necrotic areas. Congestion and reticulo-endothelial hyperplasia only was seen in one animal.

Examination of the untreated survivors and streptomycin-treated animals killed on the 12th day revealed only slight pathologic changes. There were a few small areas of mononuclear and lymphocytic infiltration in the liver surrounded by normal liver cells. Spleen sections showed moderate to marked reticulo-endothelial hyperplasia and subcapsular congestion. No difference could be detected in the sections from the treated and untreated animals after recovery. The liver sections of uninfected drug controls were normal, and the spleens showed only slight reticulo-endothelial hyperplasia.

DISCUSSION

Streptomycin was found to be as effective in the treatment of experimental tularemia in the white rat as it is in the white mouse. In the mouse experiments previously reported,[1] in which a small infecting dose was used, untreated animals died on the 5th and 6th days. Therapy was effective even if delayed for 72 hours after the mice were infected. In the rat experiments, on the other hand, only 25% of the animals survived when therapy was delayed for 48 hours. While this would apparently

indicate that streptomycin is less effective in the rat than in the mouse, it should be pointed out that the rats not only received a much larger infecting dose than did the mice but also less intensive therapy. Untreated rats became ill within 24 hours after inoculation and developed a bacteremia and focal inflammatory lesions in the liver and spleen. Nearly 50% of the untreated rats were dead within 72 hours. In the rat, as in the mouse,[1] monkey[5] and man,[3,8] definite improvement was apparent within 24 hours after therapy was initiated.

In the three untreated recovered rats tested, *Bact. tularense* could be cultured from the spleen 20 days after inoculation. In two, the liver cultures were positive, and in one only, the blood culture was positive. In the treated groups killed 12 and 20 days after infection all cultures were negative. The absence of latent infection in the treated rat may be due, at least in part, to the greater natural resistance of this species. In the rat, as in the mouse, therapy eliminates *Bact. tularense* first from the blood and then from the liver. However, in the treated mouse, in contrast with the rat, the spleen was shown to harbor the organisms for as long as 50 days after infection. In the rat experiments, while elimination of *Bact. tularense* was based on the culture of tissue smears on GCBA instead of on the injection of macerated spleens into mice, as described in paper I of this series, in another paper[4] we have shown, by the injection of ground spleens, that untreated rats usually eliminated the organisms completely within 28 days after inoculation.

It is of interest to note that untreated recovered rats were immune to challenge inoculation 20 days after infection, and that they still harbored the organisms in their spleens.

In the two experiments described in this paper, rats received 10,000 and 20,000 μg of streptomycin per kg per day. This is one-fourth to one-eighth less than the minimal effective dose in mice described in the previous paper and more in the range which has been shown to be effective in the monkey and in man.[3,5,8]

SUMMARY

1. Streptomycin was not toxic to rats in doses of 20,000 μg per kg of body weight when administered in two doses of 10,000 μg per kg every 12 hours for 10 days.

2. Streptomycin was effective in the treatment of experimental tularemia in the rat. When therapy was delayed for more than 24 hours after infection, however, a large percentage of the animals succumbed.

3. Rats recovered from infection after therapy with streptomycin were immune when challenged 20 and 35 days after the initial infection.

4. The elimination of *Bacterium tularense* from the blood, liver and spleen of streptomycin-treated animals was more rapid than in untreated survivors.

8. Howe, C., Coriell, L. L., Ellingson, H. J. and Bookwalter, H. L. 1946, Streptomycin treatment in tularemia. J.A.M.A. **132**: 195–200.

STREPTOMYCIN STUDIES IN TULAREMIA. III. STREPTOMYCIN THERAPY IN MONKEYS

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Since tularemia in monkeys has many characteristics of the disease in man,[1,2] this study was designed to observe the effect of streptomycin on the various clinical and laboratory manifestations of the disease. These manifestations can be readily measured in the monkey, whereas in the mouse or rat it is difficult to make adequate clinical and laboratory observations.[3,4] The experiment was planned to show the possible contrast between the course, outcome, laboratory findings, antibody response and pathologic picture in treated and untreated monkeys.

MATERIALS AND METHODS

The animals selected were young *Macaca mulatta* weighing 1.9 to 2.5 kg each. Rectal temperatures were taken 4 times a day at 2 A.M., 8 A.M., 2 P.M. and 8 P.M. for 20 days following infection, and at 8 P.M. daily therafter. Complete blood counts, clotting time, prothrombin time[5] and blood cultures were made daily. Blood cultures were made by spreading 0.1 ml of fresh whole blood over the surface of a glucose-cysteine-blood-agar (GCBA) plate.[6] Agglutinin titers were determined at 2-day intervals. All animals were weighed on alternate days. Urinalyses were made at suitable intervals.

Streptomycin hydrochloride* was administered by intramuscular injection of 1250 μg per kg of body weight every 3 hours, starting 48 hours after the monkeys were infected. This dosage was selected because it was midway between that reported to be effective in human tularemia by Foshay[7] and that effective in cases treated at this installation.[8] The drug was dissolved in distilled water so that each ml of solution contained 3000 μg of streptomycin base.

The method of Forgacs & Kornegay[9] was used to determine streptomycin blood levels. Preliminary studies showed that after a single intramuscular injection of 1250 μg per kg, levels of 2 to 3 μg per ml of blood were obtained within 15 minutes after injection, and a level above 1 μg per ml was maintained for 2 to 3 hours. Blood specimens taken within one-half hour after injection of the 8:00 A.M. dose of drug were used to determine the maximal blood level. The minimal blood level was ascertained from blood drawn just before the next injection of the drug† (mini-

1. Downs, C. M., Coriell, L. L., Pinchot, G. B., Maumenee, E., Klauber, A., Chapman, S. S. and Owen, B. J. Studies on tularemia. I. The comparative susceptibility of various laboratory animals. J. Immunol. In press.
2. Coriell, L. L., King, E. O. and Smith, M. Studies on tularemia. IV. Immunization of monkeys. To be published.
3. Chapman, S. S., Downs, C. M., Coriell, L. L. and Kowal, S. F. 1949, Streptomycin studies in tularemia. I. In vitro and in vivo effect of streptothricin and streptomycin on *Bacterium tularense* with special reference to streptomycin therapy in white mice. J. Infect. Dis. **85**: 25–38.
4. Chapman, S. S., Coriell, L. L. and Kowal, S. F. 1949, Streptomycin studies in tularemia. II. Streptomycin therapy in white rats. J. Infect. Dis. **85**: 39–44.
5. Smith, H. P., Warner, E. D. and Brinkhause, K. M. 1937, Prothrombin deficiency and bleeding tendency in liver injury. (Chloroform injury.) J. Exper. Med. **66**: 801–804.
6. Downs, C. M., Coriell, L. L., Chapman, S. S. and Klauber, A. Cultivation of *Bacterium tularense* in embryonated eggs. J. Bact. In press.

* We wish to thank Merck and Company, Rahway, New Jersey, for a generous supply of streptomycin hydrochloride used in these studies.

7. Foshay, L. and Pasternack, A. 1946, Streptomycin treatment of tularemia. J.A.M.A. **120**: 393–398.
8. Howe, C., Coriell, L. L., Ellingson, H. V. and Bookwalter, L. H. 1946, Streptomycin treatment in tularemia. J.A.M.A. **132**: 195–200.
9. Forgacs, J. and Kornegay, G. B. 1946, Studies on streptomycin. I. Assays in body fluids. J. Lab. & Clin. Med. **31**: 514–522.

† We are indebted to Grey B. Kornegay and Joseph Forgacs for the determination of the streptomycin blood levels on these animals.

mal levels were usually determined every other day). A detailed report of the blood levels in these monkeys has been included in another publication on the concentration of streptomycin in the blood and urine of man and animals.[10]

EXPERIMENTAL

Twelve monkeys were divided into three groups as shown in table 1. Ten animals were inoculated subcutaneously on the abdomen (below and to the left of the umbilicus) with 1 ml of a 10^{-4} dilution of a standard suspension of *Bacterium tularense*, strain Schu, containing 217,000 organisms. This dose was 10,000 to 100,000 MLD as shown by previous titration in monkeys of the same weight.[1] Five of the 10 test monkeys were retained as controls, and 5 were treated with streptomycin, beginning 48 hours after inoculation of the bacterial suspension. Two uninfected animals were used as drug controls and received the same daily dosage of streptomycin as the treated test animals. Three of the treated animals and one drug control received continuous therapy for 9 consecutive days. One treated monkey and one drug control received continuous therapy for 16 consecutive days. One of the treated monkeys received drug for 9 days, and because of a recurrence of fever, therapy was resumed for an additional 7 days.

TABLE 1.—*Data on monkeys showing the effect of treatment with streptomycin.*

Group and number	Days of therapy	Day on which a finding first became positive							Day of death
		Local lesion			Regional lymph node enlargement	Positive blood culture	Leucopenia	Petechiae	
		Edema	Hemorrhage	Necrosis					
Infected*									
65	None	2	None	None	4	3	4	4	4
56	None	2	3	3	2	2	4	4	4
17	None	2	3	4	2	3	5	4	4
62	None	2	4	5	2	3	4	5	5
54	None	2	3	4	2	4	5	None	6
Infected									
63	9†	2	None	None	2	2	None	None	Survived
57	9	2	4	5	3	None	None	None	Survived
66	9	2	None	None	2	2	None	None	Survived
71	16	2	4	4	2	None	None	None	Survived
67	9 and 7‡	2	4	6	2	None	None	None	Survived
Uninfected									
61	9	None	None	None	None	None	None	None	Survived
49	16	None	None	None	None	None	None	None	Survived

* All animals except no. 61 and 49 were inoculated subcutaneously with 1 ml containing 10,000 to 100,000 MLD of *Bacterium tularense*.
† Streptomycin dosage: 1250 μg per kg every three hours.
‡ Therapy was discontinued after the 9th day and was resumed on the 24th day for an additional 7 days.

RESULTS

Untreated infected controls.—Observations on the disease in the 5 untreated controls are presented first. Separate case reports are given for each treated animal, since the course of the disease differed somewhat with each and in two cases the therapy was varied as indicated above.

In all control animals the onset of illness was less than 24 hours after inoculation, as shown by a temperature elevation to 104 to 106 F. The temperature remained elevated until 12 to 24 hours before death, when it fell to subnormal levels. After the first day the animals were listless and became progressively weaker. Prostration occurred 12 to 24 hours before death.

The site of inoculation on the ab-

10. Kornegay, G. B., Forgacs, J. and Henley, T. F. 1946, Studies on streptomycin. II. Blood levels and urinary excretion in man and animals. J. Lab. & Clin. Med. **31**: 523–534.

domen was surrounded within 24 hours by an area of erythema measuring 0.1 to 2 cm in diameter. During the second day, subcutaneous induration and edema just to the left of the umbilicus was observed in all animals, and edema extended down to the symphysis. The regional lymph nodes were enlarged on the second day in 4 animals and on the fourth day in the remaining animal. Central hemorrhage into the skin over the indurated mass to the left of the umbilicus, followed by necrosis and ulceration, occurred in 4 of the 5 controls. Multiple petechial hemorrhages of the face, shoulders, abdomen and hind legs occurred in 4 of the 5 control animals. This is illustrated in figures 1and 5.

The laboratory findings in the control animals were quite uniform and consisted of an initial neutrophilic leucocytosis followed by marked leucopenia with almost complete absence of neutrophilic leucocytes during the 12 to 24 hours preceding death. Blood cultures became positive between the second and fourth days, and a daily logarithimic increase in the number of bacteria per ml of blood was observed. Clotting and prothrombin times were not significantly altered. Serum agglutinins were not demonstrated in these animals before death.

Table 1 presents the significant clinical and laboratory data on the control and treated monkeys. Two representative charts of the control monkeys (figures 2 and 3) show the typical changes in temperature, weight, blood culture, white blood cell count, size of the regional lymph nodes and serum agglutinin titers. Figures 4 and 5 show the appearance of the local ulcer on the third and fifth days after infection in untreated monkey number 54.

Gross pathological findings at autopsy consisted of dehydration, emaciation and multiple petechial hemorrhages in 4 of the animals. The local ulcers consisted of a thickened edematous area 4 to 6 cm in diameter, with central ulceration measuring 2 to 3 cm in diameter. The subcutaneous tissue and muscle layer were edematous, but necrosis did not extend into the muscle layer except in one animal which had a subcutaneous abscess without superficial ulceration. In all animals, the regional lymph nodes were enlarged, hemorrhagic and many were necrotic. The spleens were enlarged two to three times normal size, the edges were rounded and the capsule was smooth and bluish-gray in color. The cut surface was firm, and in one animal which lived 6 days, numerous minute yellowish focal areas were visible. The livers were swollen and showed cloudy swelling. The cut surface was mottled and pale. The adrenal glands were pale, and in one monkey the cut surface showed

FIG. 1.—Untreated monkey 56 (photographed at death) showing multiple petechiae.

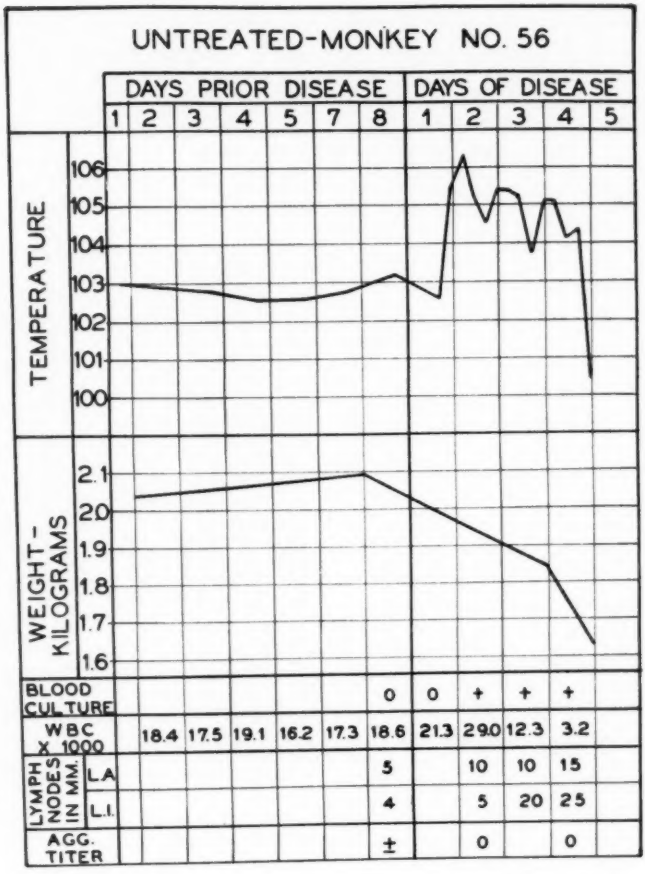

FIG. 2.—Chart of monkey 56. This untreated monkey died on the 4th day of disease. Note marked temperature rise, positive blood culture, acute lymphadenopathy and loss in weight. Leucocytosis apparent at time of marked temperature rise was followed by a progressive leucopenia until death.

L. A. and L. I. on the chart refer to the left axillary and left inguinal lymph nodes, respectively.

numerous petechial hemorrhages. The kidneys were not abnormal. The lungs and intestines were normal except in one monkey, which showed many small hemorrhages on the serous surfaces.

At autopsy various organs were cultured on GCBA plates. Positive cultures were obtained from heart blood, liver, spleen, regional lymph nodes, local lesion and, in the two cases tested, from the bile.

Microscopic examination showed coagulation necrosis of the skin at the site of the local lesion and of the regional lymph nodes. Spleen sections showed depletion of Malpighian bodies with extensive coagulation necrosis of the red pulp. There was marked fatty degeneration of liver cells together with congestion, focal hepatitis, fibrin deposition and fragmentation of polymorphonuclear and mononuclear leucocytes

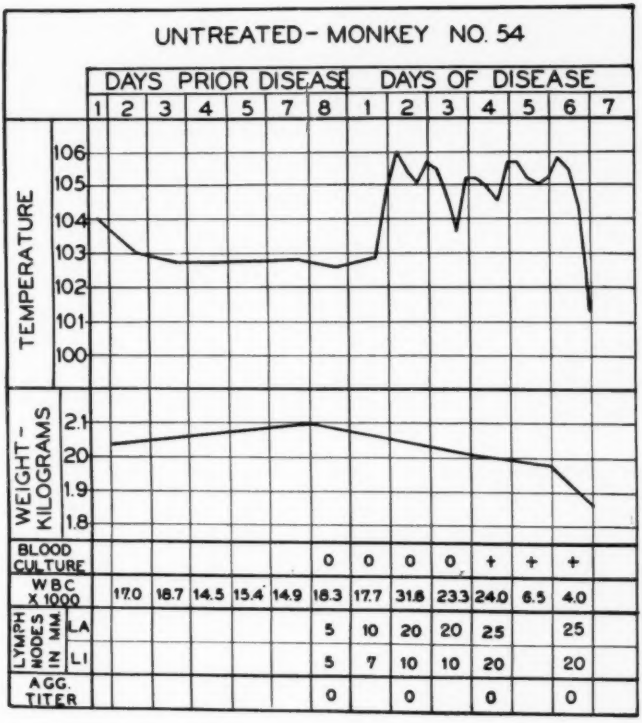

FIG. 3.—Chart of monkey 54. This untreated monkey died on the 6th day of disease. Note elevated temperature, slight loss in weight, positive blood culture, leucocytosis followed by leucopenia prior to death, acute lymphadenopathy and negative agglutinin titer.

about the areas of focal hepatitis. In some slides stained with Giemsa, large liver cells filled with *Bact. tularense* were observed. The adrenal glands showed depletion of cortical lipoid with numerous small thrombi and small foci infiltrated with polymorphonuclear and mononuclear leucocytes. The lungs showed no changes other than chronic inflammatory reaction around encapsulated parasitic mites.

Monkeys treated with streptomycin.—Five monkeys were infected at the same time as the controls and streptomycin treatment was begun 48 hours after infection, as previously described. Prior to therapy, all monkeys in this group showed a clinical course of disease similar to that observed in the control animals. A brief description and chart of each monkey are presented below.

Monkey 63, figure 6: Forty-eight hours after infection and immediately before therapy was begun, a positive blood culture was obtained, the left axillary lymph nodes were enlarged, the site of inoculation was indurated and edematous and the animal was weak and listless. Response to therapy was rapid. Most striking was the improvement evident by the second day of therapy in the general condition and activity. At this time the blood culture was negative. On the third day of therapy the temperature returned to normal and the size of the regional lymph nodes had receded on the 6th day. No marked weight loss was observed.

The differential blood count showed a shift to

FIG. 4 and 5.—Development of skin lesion in untreated monkey 54.

FIG. 4.—Photographed three days after infection. Note edema in region of umbilicus, with central hemorrhage.

FIG. 5.—Photographed 5 days after infection showing necrosis to the left of the umbilicus with edema extending to symphysis and marked adenopathy of the left inguinal node. *Bact. tularense* was recovered from the exudate in the necrotic area.

the left during the first week of disease. The total leucocyte count and differential blood count had returned to normal by the end of therapy.

Hemorrhage and necrosis of the skin did not occur and the abdominal edema disappeared after 6 days of treatment. Yellow discoloration and flaky desquamation of the skin surrounding the site of inoculation occurred on the 7th day. The agglutinin titer first became positive on the 6th day of disease and was 1:160 on the 12th day when the animal was killed. Streptomycin blood levels varied between 0.5 and 5.6 μg per ml of blood throughout the treatment period. Treatment was discontinued on the 11th day, and the animal was killed the following day.

At autopsy, a fibrotic subcutaneous nodule measuring 1 cm in diameter was observed to the left of the umbilicus. The regional lymph nodes were slightly enlarged but not necrotic. The liver, lungs and adrenal glands appeared normal. The spleen was normal in size and texture but a few pinpoint white spots could be seen through the capsule.

Microscopic examination of the subcutaneous nodule showed active connective tissue infiltrated with numerous macrophages, lymphocytes and amorphous debris. The lymph nodes showed follicular hyperplasia and connective tissue reaction in the sinusoids. In the liver the most prominent finding was diffuse fatty infiltration throughout the lobules. A few foci of periportal mononuclear cells were observed. The spleen showed only lymphoid hyperplasia. Other organs were not abnormal.

Monkey 57, figure 7: Pre-therapy clinical observations were the same as those in the untreated series. The blood cultures were not positive at any time in this animal. By the second day of therapy, the animal showed general improvement, ate well and appeared in good condition despite the elevated temperature, which did not return to normal until the 4th day. Dehydration disappeared within two days and the monkey continued to gain weight.

Marked leucocytosis and shift to the left present on the day therapy was begun was followed by a mild leucopenia and normal differential count for the first three days of therapy. Thereafter the blood picture was essentially normal. Eosinophiles which disappeared during the height of the febrile stage reappeared after two days of therapy.

Regional lymphadenopathy of the left axillary node persisted during the 9 days of therapy but disappeared by the 17th day after infection. The left inguinal node had become normal by the 5th day of treatment. Biopsy of this node 21 days after infection showed it to be of normal size and consistency. Microscopically only hyperplasia was present. Mice inoculated with macerated tissue from this node remained well.

Before therapy was commenced, the local lesion was seen as an erythematous and edematous area to the right of the challenge site. This area became indurated and hemorrhagic, and central necrosis occurred on the 5th day. Edema extended over the lower abdomen. A crust had formed by the 7th day and exudate removed from beneath it was positive for *Bact. tularense*

At autopsy there was no scarring or induration of the subcutaneous tissue. All lymph nodes were small, firm and slightly fibrotic. The parenchymatous organs were normal in size and appearance. No *Bact. tularense* was recovered from mice injected with macerated liver, spleen, lung, bone marrow, bile and regional lymph nodes. Microscopically the local lesion showed slight

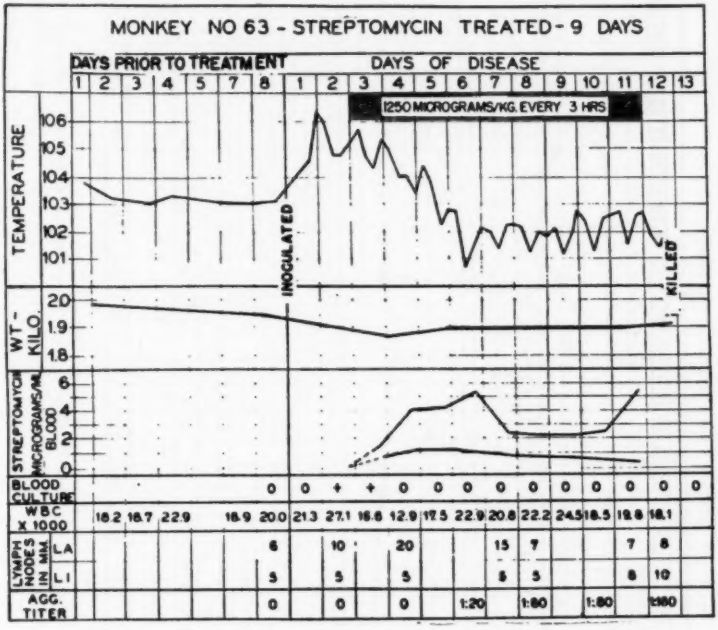

FIG. 6.—Chart of monkey 63. This animal was treated for 9 days and killed at the end of therapy. Under treatment the temperature returned to normal and there was no appreciable loss in weight. The blood culture which was positive at onset of therapy was negative after one day of treatment. The total leucocyte count returned to normal after one day of therapy. Left axillary lymphadenopathy disappeared after 5 days of treatment. Within 5 days after infection the agglutination test became positive.

on the 7th but negative on the 9th day of therapy. Cultures were also negative when made from this site 10 days after the cessation of therapy. By the 39th day, when the animal was killed, only a small scar remained at the site of the local lesion.

The agglutinin titer was 1:20 six days after infection, increased on the 12th day to 1:160, and was maintained at a level of 1:320 from the 27th day until the animal was killed. Streptomycin blood levels ranged between 1.2 and 8.0 μg per ml.

infiltration of lymphocytes in organizing fibroblastic tissue. The overlying epithelium was normal.

No pathological change was present in the lymph nodes, lungs, spleen, kidney or adrenal. There was diffuse fatty infiltration of the liver.

Monkey 71, figure 8: Prior to the initiation of therapy this monkey was ill as shown by its general appearance. Its temperature rose sharply between the 18th and 24th hours after infection and was 105.8 F at the 30th hour. Regional lymphadenopathy was evident. The characteris-

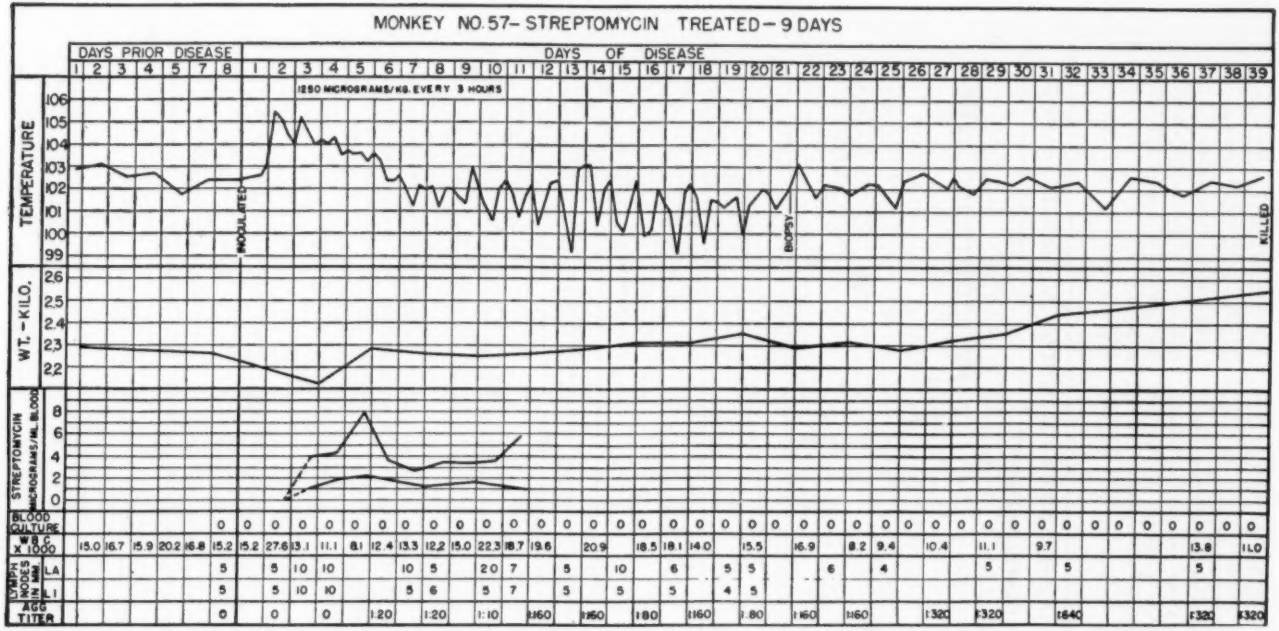

FIG. 7.—Chart of monkey 57. This animal was treated for 9 days. Note the drop in temperature during streptomycin therapy. There was no appreciable weight loss. Slight leucocytosis evident at time of onset of treatment was followed by a normal leucocyte count. Lymphadenopathy disappeared under treatment. This monkey developed agglutinins by the 6th day after infection. The left inguinal lymph node biopsied 21 days after infection was negative when cultured for *Bact. tularense*. Cultures at death were negative.

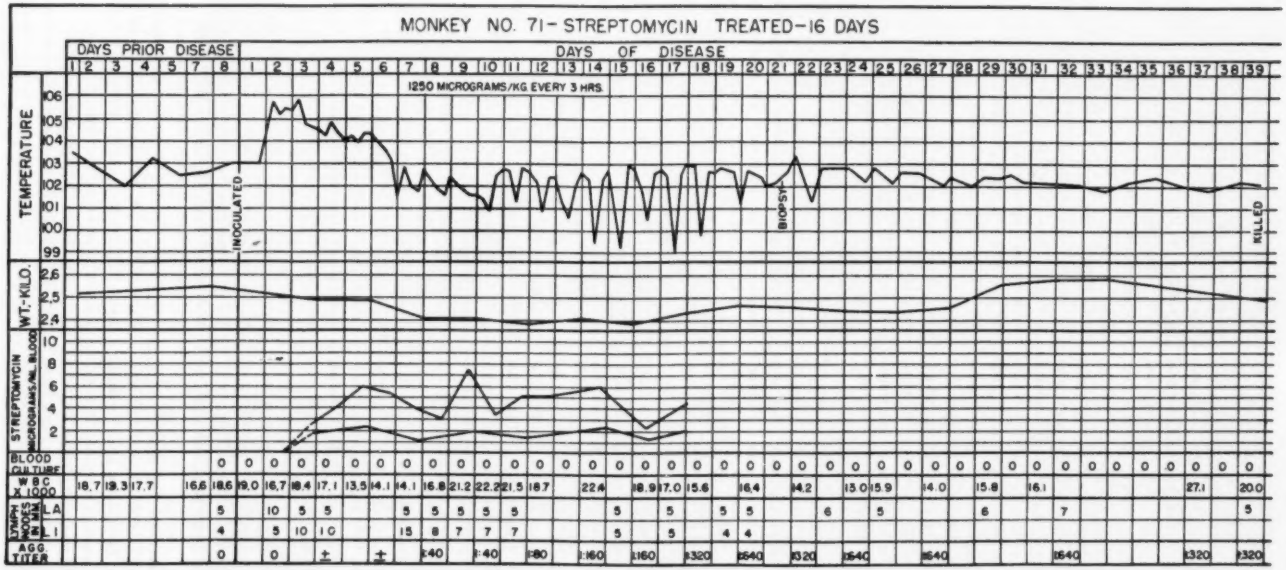

FIG. 8.—Chart of monkey 71. This animal was treated for 16 days and did not have recurrence of fever after treatment was discontinued. The temperature returned to normal after 4 days of treatment. Lymphadenopathy of left axillary and left inguinal nodes disappeared. During treatment the weight change was slight and agglutinins developed by the 8th day. At the time of death no *Bact. tularense* could be cultured from any tissue. The local lesion healed readily (see fig. 9–12).

tic leucocytosis was absent on the second day after inoculation but there was a slight shift to the left. Within one day after therapy was started the animal became active, ate well and was markedly improved. Dehydration and general malaise disappeared after 2 days of therapy, and the temperature returned to normal after 4 days of therapy. The eosinophiles reappeared on the 5th day of the disease, and at this time there was a shift to the left which persisted for approximately 10 days. The blood picture then returned to normal. Adenopathy of the left axillary nodes disappeared one day after therapy was instituted. The left inguinal node became normal in size on the 15th day and was biopsied on the 21st day of the disease. No necrosis was evident and *Bact. tularense* was not recovered by mouse inoculation. The blood cultures were negative at all times during the course of the experiment. The local skin lesion, which was the most severe of all those present in the treated series, appeared within 24 hours as a small erythematous area at the site of inoculation. Edema and erythema increased, and by the third day induration was marked. Hemorrhage and necrosis of the overlying skin occurred on the 4th day. There was generalized edema of the lower abdominal skin. By the 5th day after therapy, the edema decreased and the local lesion had commenced to heal. On the 9th day, when therapy was discontinued, a small healing ulcer with rolled edges and a clean base remained. At the time the animal was killed a small bluish scar was evident. *Bact. tularense* was recovered by mouse inoculation from the exudate under the crust on the 7th day of therapy. On the 9th day of therapy and 10 days after therapy was discontinued cultures were negative. Figures 9, 10, 11 and 12 show the development and healing of the local lesion in this animal.

The agglutinin titer was 1:40 on the 8th day and then rose to 1:640 on the 20th day. The titer remained between 1:320 and 1:640 thereafter. Streptomycin blood levels ranged between 1.2 to 7.5 μg per ml during the period of therapy.

At the time this monkey was killed it appeared well nourished with a completely healed local lesion. All lymph nodes were small, firm and normal in appearance on cut section. The parenchymatous organs were normal in size and appearance.

The local lesion presented the picture of a healing ulcer. A few polymorphonuclear cells and many lymphocytes and fibroblasts were present. The overlying epithelium was normal. The sections of the lymph nodes, spleen, lungs, kidneys and adrenals showed no significant deviations from normal. There was slight fatty infiltration of the liver with a few small areas of lymphocytic infiltration.

Monkey 66, figure 13: At the time therapy was started, this monkey had a bacteremia, a temperature of 106.4 F and appeared very weak. After two days of therapy it was noticeably stronger, ate well, and regained normal activity after 6 days of therapy. Blood cultures were sterile after one day, and no localized induration or ulceration developed at the site of inoculation although the skin in this region was erythematous over an area measuring 3 by 6 cm. The temperature returned to normal on the 7th day of therapy. Two days after inoculation this monkey developed a leucocytosis with a shift to the left. The total leucocyte count returned to normal after one day of therapy, but a moderate shift to the left persisted throughout the period of observation. The agglutinin titer became positive on the 6th day of disease and reached 1:2560 on the 32nd day. Streptomycin blood levels varied between 0.7 and 8.3 μg per ml of blood.

Therapy was dicontinued on the 11th day of disease. On the 14th day the temperature rose to 106 F and remained elevated until the 28th day. The right inguinal node and left axillary nodes increased in size during the period of recurrent fever but regressed spontaneously. On the 22nd to 25th days there was an increased percentage of mature polymorphonuclear leucocytes with an increase in total leucocyte count on the 23rd day. The left inguinal node was removed at biopsy on the 21st day, macerated and injected into 4 white mice. All mice survived for 15 days. This animal was killed 39 days after inoculation, and at autopsy the only abnormal finding was an enlarged right axillary lymph node. *Bact. tularense* was recovered from this lymph node by animal inoculation. The spleen and liver contained only small focal areas of large mononuclear cells and lymphocytes. Other parenchymatous tissues were normal.

Monkey 66, figure 14: Before therapy was commenced, this monkey showed the same general clinical picture previously described. Response to therapy was noticeable by the end of the first day. Slight intermittent fever persisted until the 6th day of therapy. On the day therapy was commenced there was a leucocytosis with a marked shift to the left. Within three days the total white count had returned to normal accompanied by a slight shift to the left. The size of the left inguinal nodes diminished under therapy, but the left axillary nodes remained large.

At the time therapy was started there was edema and induration at the point of inoculation. This area became hemorrhagic on the 4th day,

FIG. 9–12.—Monkey 71. Four stages in the development and healing of the cutaneous lesion under streptomycin therapy. Monkey 71 exhibited the most severe lesion observed in a treated monkey.

FIG. 9.—Photographed on the third day after infection at the time therapy was initiated. Note the marked edema surrounding the umbilicus with an enlarged right inguinal lymph node.

FIG. 10.—Cutaneous lesion photographed on the 5th day after infection showing central necrosis of the indurated area to the left of the umbilicus. The lymph nodes were normal in size at this time.

FIG. 11.—Lesion on the 9th day after infection, showing less edema and a punched out ulcer with a clean granulating base.

FIG. 12.—Photograph taken on the 14th day after infection showing continued healing of the ulcer.

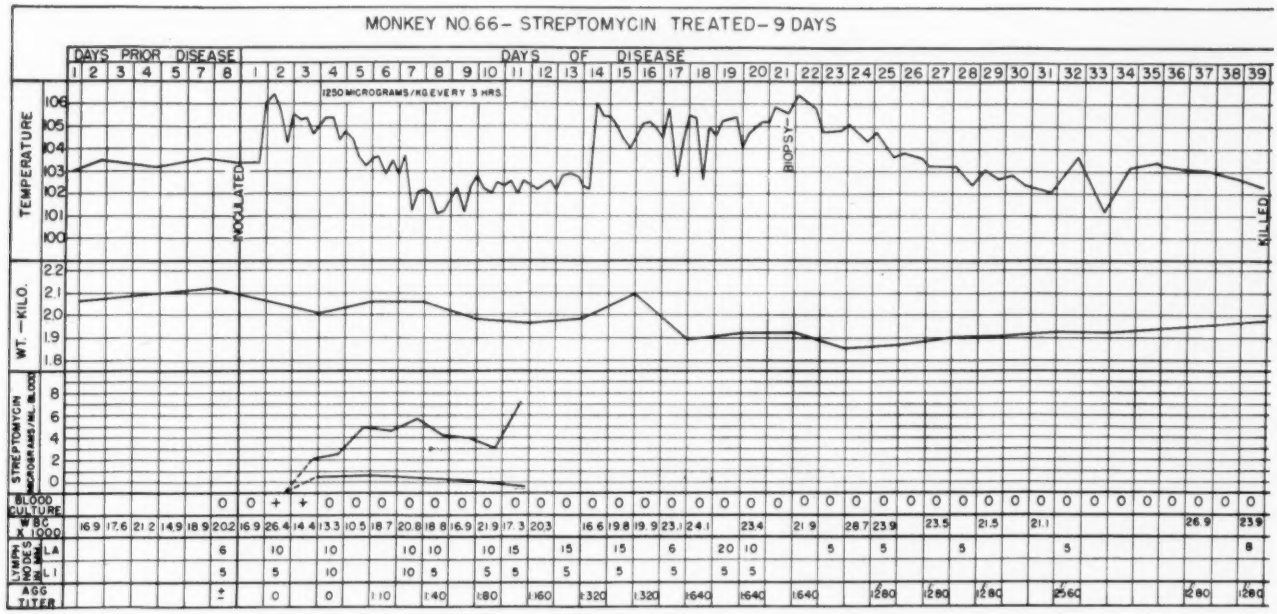

FIG. 13.—Chart of monkey 66. This animal showed good clinical recovery during a 9-day course of streptomycin therapy. The blood culture became negative and erythema and local edema at the inoculation site disappeared readily without necrosis or induration. When therapy was discontinued, the temperature was normal, the agglutinin titer was 1:160 and except for an enlarged left axillary lymph node, this monkey appeared completely recovered. Three days after therapy was discontinued the fever recurred but regressed spontaneously after 12 days. A biopsy of the left inguinal node obtained during the second febrile period was negative when cultured for *Bact. tularense*. The organism was cultured from the right axillary lymph node on the 39th day, when the animal was killed.

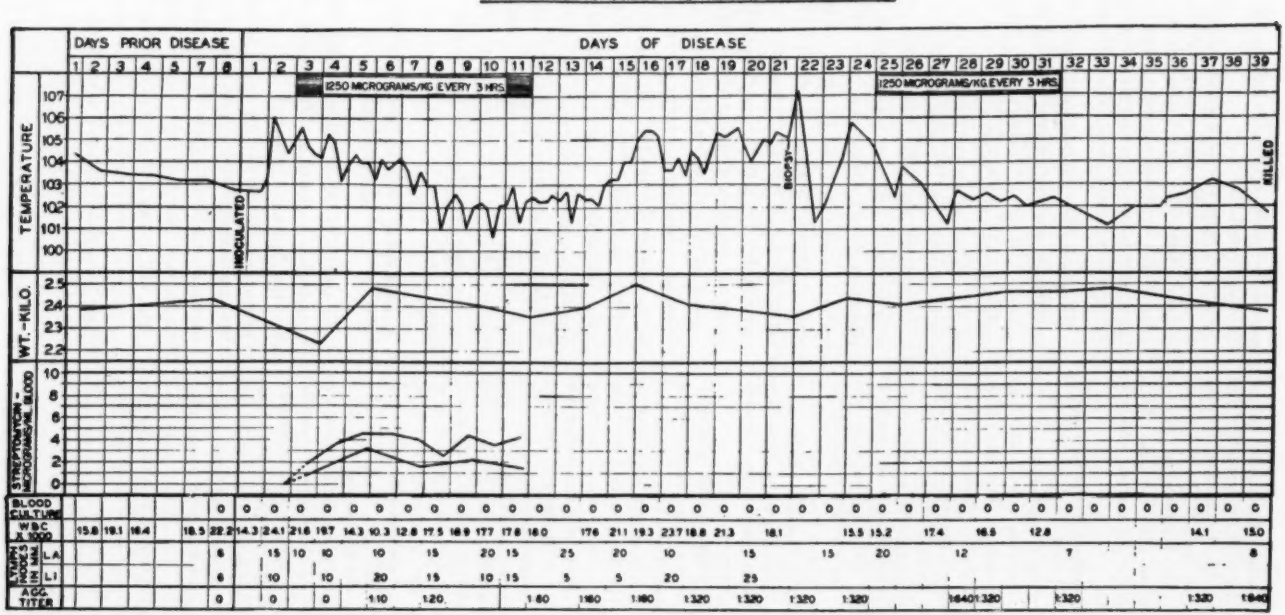

FIG. 14.—Chart of monkey 67. This monkey was treated for 9 days and appeared fully recovered at this time except for persistent enlargement of the left axillary node. Four days after cessation of therapy the fever recurred, lymph nodes became enlarged, the cutaneous ulcer became hyperemic and culture of the exudate as well as that of a biopsied left inguinal node yielded a virulent culture of *Bact. tularense*. A second course of therapy was initiated with immediate favorable clinical response. During this second course of treatment, the local cutaneous lesion and the biopsy incision became negative to culture for *Bact. tularense*. When killed 8 days after the second course of streptomycin all tissues cultured were negative.

induration increased and there was central necrosis by the 6th day with the formation of a crust on the 7th day of the disease. Healing progressed satisfactorily despite the recurrence of fever on the 15th day of the disease. Pus removed from beneath the crust contained *Bact. tularense* on the 7th day of therapy but was negative on the 9th day.

When therapy was stopped on the 11th day of the disease, the temperature and blood picture were normal, the local lesion was healing and the only visible sign of infection was an enlarged lymph node in the left axilla. Three days after the cessation of therapy the temperature rose, and lymphadenopathy of the left axillary and left inguinal regions became pronounced. Four days later the indurated area around the local ulcer was hyperemic and contained small papules. These became necrotic and *Bact. tularense* was cultured from them. The necrotic center of the original ulcer became inflamed and increased to 5 mm in diameter. During the first 5 days of recurrent fever there was an increase in total leucocyte count with a high percentage of mature polymorphonuclear leucocytes.

A biopsy of the left inguinal node was made during the recurrent fever on the 21st day of the disease. This node was markedly enlarged and necrotic, and *Bact. tularense* was recovered by mouse inoculation. The pus draining from the biopsy incision also contained *Bact. tularense*. Therapy at the same dosage was resumed in this animal on the 25th day of the disease. The local lesion showed immediate improvement; the pustules dried and sloughed and the center of the ulcer crusted over. No cultures could be obtained from node biopsy material, incision or local skin ulcer after three days of the second course of treatment. Therapy was continued for 7 days, and at the end of the time the local lesion was almost completely healed.

At no time during the disease was a positive blood culture obtained. The agglutinin titer was 1:10 on the 6th day of the disease, increased steadily to 1:640 on the 28th day and thereafter fluctuated between 1:320 and 1:640. The recurrence of fever and symptoms did not lower the antibody titer. Streptomycin blood levels during therapy varied between 1.0 and 4.6 μg per ml of blood.

This monkey was killed 39 days after inoculation. A small scar marked the site of the local lesion. The regional lymph nodes were not enlarged. The spleen, liver, kidneys, lungs and adrenals were normal in size and appearance.

The left inguinal and right axillary lymph nodes, the spleen, liver, kidney, lung, bone marrow and bile were negative for *Bact. tularense* by mouse inoculation.

Microscopically the local lesion showed more diffuse cellular infiltration throughout the subcutaneous layer than was observed in the other animals. The overlying epithelium was normal. The parenchymatous tissues were normal except that the liver showed slight fatty infiltration.

Streptomycin control monkeys.—Two uninfected animals were treated as described above. Monkey 61 received streptomycin for 9 days and was killed on the 10th, while monkey 49 received streptomycin for 16 days and was killed 3 days after the administration of the drug was stopped. No fever, weight loss, albuminuria or other evidence of drug toxicity was observed in these two animals. Blood levels of streptomycin varied between 0.9 and 8.0 μg per ml for monkey 61. Figure 15 shows the clinical chart on monkey 49 treated for 16 days.

Post mortem examination of both animals presented the same picture. The livers were yellowish in color and slightly mottled. The kidneys showed faint yellowish streaks in the cortex. All others tissues were normal. Microscopic examination showed mild fatty infiltration of the liver cells and of the epithelial cells of the kidney tubules without necrosis or inflammatory tissue reaction

Resume of clinical and laboratory observations

1. Cultures of lesions at the site of inoculation.—Exudate from the local skin lesions was injected into mice at various intervals during and after the therapy period. *Bact. tularense* was recovered on the 5th day but not on the 9th day of therapy from the 4 animals with skin ulcers. From one animal, number 67, which had a recurrence of fever, positive cultures were again obtained on the 21st day of disease. This animal was given a second course of therapy, and cultures of the local lesion

were negative on the 28th day of disease.

2. Culture of lymph nodes.—In 3 of the 4 cases treated for 9 days, in which the left inguinal lymph node was biopsied on the 21st day after infection, no *Bact. tularense* could be recovered by mouse inoculation. Similar material from monkey 67 taken on the 21st day cultures 48 hours after infection. Three other controls had positive blood culture 72 hours after infection, and the 5th control was positive 96 hours after infection. The remaining treated animals did not give positive blood cultures.

4. Urinalysis.—During the acute phase of the disease, untreated and treated monkeys showed mild albumi-

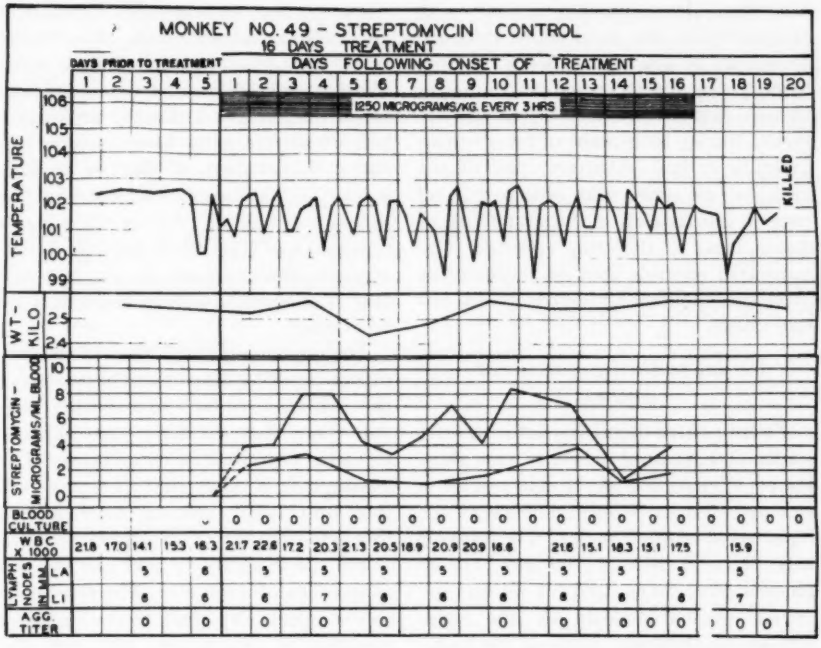

FIG. 15.—Chart of monkey 49. This animal was not infected but was treated with 1250 μg per kg every 3 hours for 16 days. Clinically there was no visible effect traceable to drug administration.

contained virulent organisms, but all nodes injected into mice at autopsy 18 days later were sterile. The biopsied left inguinal node of monkey 66 was negative on culture during the stage of recurrent fever. At autopsy 18 days later, however, the right axillary node yielded a positive culture.

3. Blood cultures.—One control and two treated animals had positive blood

nuria with a few hyaline casts. Urine findings were otherwise negative.

5. Hematology.—Clotting and prothrombin times were not significantly changed throughout these studies. Hemoglobin per 100 ml of blood dropped from an average of 13 gm to 8.5 to 10 gm during the course of observation in both treated and untreated animals. In the animals killed on the

39th day the hemoglobin values had returned almost to normal. The total red blood cell count paralleled the hemoglobin. On the second day after infection all animals showed total white blood cell counts of 18,000 to 31,000 per cmm with 70 to 85% mature neutrophiles. In animals which died of infection the total count fell to 3000 to 4000 per cmm before death, with 3 to 24% neutrophiles. In animals treated with streptomycin, the total count returned to normal in less than a week. A brief leucocytosis and increased percentage of mature neutrophilic leucocytes was observed during recurrence of fever in two animals. A few immature cells of the granulocytic series were observed in all treated animals after the first week of disease and in the drug controls. The untreated controls showed almost complete disappearance of neutrophiles before death.

Dermal sensitivity of recovered monkeys

Twenty-three days after infection the 4 surviving, treated monkeys were skin-tested. Preparation of the skin test antigen and tests on 29 normal monkeys are reported in another paper.[3] The antigen was diluted 1:1000, 1:100 and 1:10 in physiological saline, and 0.1 ml was injected intracutaneously on the right side of the abdomen. None of the monkeys showed a positive test to the 1:1000 dilution of antigen, which is routinely used in detecting hypersensitivity in man. Two monkeys showed doubtful reactions to the 1:100 dilution, while 3 of the 4 animals showed positive reactions to the 1:10 dilution.

DISCUSSION

The therapeutic efficacy of streptomycin in monkeys with tularemia was dramatically apparent in these studies. The prompt improvement in the general appearance of the monkeys, the return to normal temperature, healing of the local lesion, regression of lymphadenopathy, disappearance of septicemia and rise in antibody titer were very striking. In contrast, the disease was rapidly fatal in untreated control animals. The monkey has proved to be a more satisfactory animal than the mouse or rat in the investigation of chemotherapy of tularemia. In addition to death as an endpoint, the monkey offers an opportunity to follow many clinical and laboratory manifestations of the disease which closely simulate the picture seen in man. There is some evidence that 9 days of therapy at 1250 μg per kg was insufficient since in two monkeys under this dosage there was a recurrence of fever three days after therapy was discontinued. In the one animal treated for 16 days there was no recurrence.

Foshay has reported[7] that in streptomycin-treated human cases of tularemia the rise in antibody titer during convalescence does not differ from that observed in untreated recovered cases. The assumption is made that in man streptomycin therapy does not interfere with the development of immunity, insofar as the rise in antibody titer can be used as an index of immunity. The same conclusion appears to apply to the monkeys reported in this paper, although the animals were not challenged to measure the degree of immunity following recovery. However, one monkey which relapsed made a spontaneous recovery without further treatment, and it can be assumed that this animal acquired some immunity during the primary infection while receiving streptomycin.

The treated monkeys developed dermal sensitivity following recovery, and in this respect also they resemble man, although the degree of sensitivity is of a lower order, as shown by the amount of

antigen necessary to elicit a positive reaction.

There were no clinical signs of toxicity from the drug. The slight lipoid deposition in the liver and kidney observed by us has been observed and studied more extensively by others[11] and does not appear to be a contraindication to the use of streptomycin.

The material presented here also shows that it was not difficult to attain blood levels above those required to kill the organisms in vitro. Effective levels were maintained by small doses (10,000 μg per kg per day) when compared to the amounts of streptomycin which have been used in treating other diseases in man.[12]

SUMMARY

1. In five monkeys infected with *Bacterium tularense* the disease ran a fulminating course and terminated in death between the 4th and 6th days after infection.

2. Five monkeys similarly infected were treated with streptomycin starting 48 hours after infection. The therapeutic effects were promptly manifest and all treated animals survived.

3. Two uninfected control animals which received streptomycin showed no toxic effects which could be considered a contraindication to the use of the drug.

4. Clinical, laboratory and pathological data on the course of tularemia in the monkey are presented.

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THE CONCENTRATION OF MUMPS VIRUS

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Efforts to improve the purity and immunizing potency of influenza vaccines have encouraged the development of a variety of methods for concentration and purification of influenza virus. Attempts have been made[1,2,3] to vaccinate human beings against mumps with unconcentrated viral preparations, and one experimenter has described his efforts to concentrate the virus for the immunization of monkeys against experimental mumps infections.[4] There is little doubt that from concentrated mumps virus a more effective immunizing agent can be produced. Since egg-adapted strains are now available, the methods found successful in concentration of influenzal virus suggested themselves. Two methods have been employed in this study, namely, the alcohol precipitation procedure recently reported by Cox et al[5] and the widely used embryonic red-cell method of Francis and Salk.[6] Interesting points of difference in the behavior of mumps and influenza viruses were encountered which necessitated some changes in the procedure described by the last-named authors.

MATERIALS

Dr. John F. Enders, of Harvard Medical School, was kind enough to furnish us with the strain of mumps virus which we have used. This was an amniotic fluid representing the 39th passage in eggs. Two allantoic passages were sufficient to accustom it to that route of inoculation, and it has been maintained subsequently by allantoic inoculation of 6- or 7-day embryonated eggs. After further incubation for 5 to 7 days the harvested allantoic-amniotic fluids usually agglutinated chicken red cells in titers ranging from 1/320 to 1/1280 and have provided the virus preparations used in this study.

EXPERIMENTAL

Concentration by means of methyl alcohol

In the concentration of mumps virus by alcohol precipitation the procedure outlined by Cox and his co-workers[5] for influenza virus was followed with minor modifications only. To equal fractions of pooled allantoic-amniotic harvest, reagent grade methyl alcohol was added in steps of 3% between 9% and 30% final concentration. The virus was chilled beforehand to about 1 C and the methyl alcohol to about −10 C. The mixtures of the two were allowed to stand at −5 to −10 C for 2½ hours to facilitate maximum precipitation with as little denaturation as possible. The precipitate was then thrown down at a speed of 3500 r.p.m. for 30 minutes in the angle head of a refrigerated centrifuge set at 20 F (−5 C). The supernatants of each fraction were discarded, and the precipitates were re-dispersed in a quantity of nutrient broth (or in a few instances, for comparative purposes, other diluents) equal to one-tenth the original volume.

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Table 1 shows the agglutination titers against chicken red cells of the concentrates resulting from precipitation by percentages of methanol from 9% to 30%. Although the results are not perfectly uniform in successive experiments, it can be seen that recovery of the virus is most complete when 18% to 21% alcohol is used. As reported by Cox and his associates[5] in their concentration of influenza virus, recovery appeared to exceed 100% in some instances if the relative titers of unconcentrated virus and 10-fold concentration of the same can be accepted as a criterion. This interesting phenomenon is being studied further.

Cox and his co-workers[5] showed that the infectivity of influenza virus was not appreciably altered by the percentage of alcohol which was most effective in concentration. The same is probably true for mumps virus concentrated by precipitation with 20% methanol. While the minimal infective dosage of such concentrates for embryonated eggs has not been determined, 10^{-3} or 10^{-4} dilutions have consistently infected eggs inoculated therewith.

TABLE 1.—*Concentration of mumps virus by methyl alcohol precipitation.*

Harvest		CCA titers of fractions concentrated ×10 by alcohol precipitation							
Date	CCA titer	9% alcohol	12% alcohol	15% alcohol	18% alcohol	21% alcohol	24% alcohol	27% alcohol	30% alcohol
12-5	640			2,560	5,120	5,120	2,650	1,280	1,280
12-19	1,280			20,480	20,480	20,480	10,480	5,120	2,560
12-30	640		20,480	10,240	5,120	2,560	2,560		
1-6	160	640	2,560	2,560	5,120	2,560	2,560		
1-21	640	2,560	5,120	5,120	10,240	20,480	5,120		
4-28	640	1,280	2,560	5,120	5,120	5,120	5,120		

TABLE 2.—*Red cell adsorption as a means of concentrating mumps virus.*

Harvest		CCA titers after adsorption with							
		2% chicken RBC		2% human RBC†		2% guinea pig RBC		2% sheep RBC	
Date	CCA Titer	Eluate	Supernatant	Eluate	Supernatant	Eluate	Supernatant	Eluate	Supernatant
12-4		40*	640						
12-18	1,280	160	640	40	640				
12-23	640					320	320	10,240	80
12-30	640					1,280	320	10,240	160
1-13	640	160	640			320	640	2,560	320
2-3	160			20	160	80	160	640	80

* Embryonic red cell method used.
† Group 0.

Adsorption on and elution from 2% erythrocyte suspensions

In our first attempts at concentration by adsorption on and elution from chick embryo red cells the technic of Francis and Salk[6] was followed without important modification. To test the adsorptive capacity of the red cells of adult chickens and of other species, however, it became necessary to chill the eggs several hours before harvesting in order to eliminate the escape of embryonic erythrocytes into the allantoic fluid. To separate fractions of the pooled harvest, red cells of several species were added in a final concentration of approximately 2%, which is about the maximum density of embryonic red cells achieved by slashing the allantoic blood vessels in the method of Francis and Salk. Whole blood was used at first and was assumed to have a 50% red cell content. In most of the experiments, however, washed, packed, and measured red cells were added to a 2% concentration. The harvest with red cells added was then chilled for 45 minutes at a temperature of 1 to 2 C (ice-bath) before centrifugation in a 5 C cold room. The rest of the

TABLE 3.—*Effectiveness of heavier erythrocyte suspensions (chicken and sheep) in the adsorption-elution procedure.*

Adsorption		CCA titers following adsorption and elution*											
RBC used	CCA titer of unconcentrated virus	2% RBC		5% RBC		10% RBC		15% RBC		20% RBC		30% RBC	
		Eluate	Supernatant	Eluate	Supernatant	Eluate	Supernatant	Eluate	Supernatant	Eluate	Supernatant	Eluate	Supernatant
Chicken no. 4	320	160	160	160	80			160	80			40	80
Sheep no. 2		2,560	40	2,560	<20			2,560	<20			1,280	<20
Sheep no. 4	640	2,560	80	>10,240	40			5,120	<20			5,120	<20
Sheep no. Gi		5,120	80	>10,240	40			10,240	—			5,120	—
Chickens no. 8 & no. 9 (pooled)	320	640	320	1,280	160	2,560	80	2,560	80	2,560	80		
Sheep no. Gi		1,280	160	5,120	160	5,120	40	5,120	40	10,240	20		

* In each case elution was made into Difco nutrient broth (one-tenth original volume).

procedure followed the Francis-Salk technic except for the use of Difco nutrient broth as an elution fluid. The eluate volume in each instance was one-tenth that of the original fraction of the pooled harvest.

The good hemagglutination titers obtained when allantoic mumps virus was titrated against chicken red cells encouraged us to expect that concentration could be accomplished by the procedure of Francis and Salk,[6] but repeated attempts have shown this not to be the case. The results of one of these efforts are shown in table 2. Somewhat more favorable results were obtained in some instances when washed and measured adult chicken cells were added for adsorption purposes, but recovery (by elution) was never good enough to recommend the method for concentration of the virus. (See tables 2 and 3.)

Since it is well known that the erythrocytes of a number of mammalian species adsorb influenza virus (at least enough to be agglutinated in the process), the red cells of several easily available species were tested both for agglutinability in mumps virus and for capacity to adsorb substantial amounts of the same. The red cells of guinea pigs and man are agglutinated in essentially the same dilutions of this virus as are chicken cells and appear to be little or no better for concentration purposes than the latter (see table 2). The cells of all three species show the same agglutination pattern as that caused by influenza virus.

The red cells of sheep can only be described as unpredictable as far as agglutination by mumps virus is concerned until the conditions which determine such agglutination are better understood. They seem consistently, however, to adsorb enough of this virus to serve as more effective vehicles of con-

centration (see table 2) than do the red cells of the species mentioned above. No relationship could be discovered between agglutinability and adsorptive capacity; and the red cells of different sheep, tested simultaneously with different fractions of the same allantoic virus, have been found to vary considerably in their ability to adsorb and relinquish (elute) the virus. When 2% erythrocyte suspensions from 7 different sheep were so tested, the CCA titers of the several eluates ranged from 1/160 to 1/5120. The virus before adsorption possessed a CCA titer of 1/320.

The red cells of some sheep appear not to be agglutinated at all by mumps virus. Since, however, the cells of certain sheep have agglutinated at one time and subsequently have failed to agglutinate when a different lot of the same strain of virus was used, it seems possible that the cells of most or all sheep may be made to agglutinate in mumps virus under appropriate conditions. We are attempting by altering some of the physico-chemical conditions to ascertain the cause of this erratic behavior. It should be stated also that the agglutination pattern when sheep erythrocytes are used is different from that shown by chicken, guinea pig, or human cells. The clumps of cells are smaller and there is less difference in the degree of agglutination caused by high and low dilutions of the virus.

Adsorption on and elution from heavier erythrocyte suspensions

As would be expected, a higher proportion of virus is adsorbed when the density of the red cell suspensions is increased (see table 3). This is true both for chicken and sheep cells, though the latter are obviously a better adsorption vehicle at every density tested. In this table it can also be seen that the CCA titers of the supernatant fluids bear a rough inverse relationship to the titers of the corresponding eluates. While it is apparent that most of the adsorbed virus is released during the 2½ hour elution period, there is some indication that the heavier red cell suspensions hold on to measurable amounts. This can be seen if, for example, the eluate and supernatant titers following adsorption on 15% or 30% suspensions of sheep #4 red cells are compared with corresponding titers following adsorption with 5% suspensions of these cells. Eluate titers were lower when the heavier suspensions were used in spite of the fact that supernatant titers showed more virus was adsorbed.

The adsorption of mumps virus on red cells and its elution therefrom are undoubtedly influenced by the particular physical and chemical conditions under which these processes are carried on. The effect of varying some of these conditions is being studied in our laboratory and will be reported at a later date.

SUMMARY

1. The alcohol precipitation method which Cox and his co-workers have found highly satisfactory for concentration of influenza virus is equally useful for concentrating mumps virus grown in the allantoic sac of the chick embryo.

2. Mumps virus will agglutinate the red cells of chickens, guinea pigs, human beings, and sheep, the latter with less regularity. Adsorption of the virus upon the red cells of each of these species will occur but consistently in greater degree when the cells of sheep are used.

3. Enhanced adsorption of mumps virus occurs when denser suspensions of the red cells of any of these species are used, but the greater effectiveness of sheep erythrocytes as an adsorption vehicle is apparent at all densities tested (up to 30% suspensions).

RELEASE OF THE A_2 ISOAGGLUTINOGEN-LIKE SUBSTANCE OF INFECTIOUS ORGANISMS INTO HUMAN BLOOD SERUM

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Substances immunologically related to the group A substances of human blood have been isolated from various infectious organisms.[1,2] These substances as a rule are polysaccharides which have been extracted chemically from the live or dead organisms. The polysaccharide when added to human serum inhibits the aggultination of human erythrocytes. The role which these polysaccharides play in the metabolism of the parasite and in the immune and pathological conditions in the host resulting from infection are little understood at present. It has been suggested that during infections with organisms possessing this isoagglutinogen-like substance, the host may become immunized to it and that autoagglutinins may develop to the host's own erythrocytes.[3]

Recently Franks[4] has reported the presence of autoagglutinins and agglutinated red blood cells in the serum of individuals with circulating microfilariae of *Wuchereria bancrofti*. Since the isoagglutinogen-like substance has been found in various helminths and also in the cotton rat filarial parasite *Litomosoides carinii*,[5] it is logical to assume that such a substance may also be present in *W. bancrofti*. Therefore, the agglutinins described by Franks may develop as a result of immunization with the polysaccharide in the human parasite. A great number of the organisms described below, which include bacteria and helminths, apparently possess this substance. This fact may help in the understanding of the significance of the isoagglutinins present in human serums. Also, the cross reactions observed among infectious agents may be explained by the presence of the common isoagglutinogen-like factor in them.

MATERIALS AND METHODS

The helminth parasites used in this study were the larval forms of *Trichinella spiralis* and the adult forms of *Schistosoma mansoni*. Trichina larvae were obtained from the muscles of laboratory-infected rats digested in a pepsin-hydrochloric acid mixture. The adults of *S. mansoni* were obtained from the portal and mesenteric veins of experimentally infected mice 6 to 8 weeks after intraperitoneal inoculation with cercariae.

The bacteria utilized were obtained from stock cultures which were preserved at the School of Tropical Medicine, and others came from the

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The authors wish to express their gratitude to Dr. W. H. Taliaferro, of the Dept. of Bacteriology and Parasitology, The University of Chicago, Chicago, Ill. and to Dr. Arnold D. Welch and Dr. Ernest Beuding of the Dept. of Pharmacology, Western Reserve University, Cleveland, Ohio, for their valuable criticism of the work. The technical assistance of Mr. Carlos J. Thillet is also acknowledged.

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American Type Culture Collection, Washington, D. C.

Incubation of organisms in human serums of groups A and B.—Known numbers of helminths were placed in 0.2 cc of unheated human serums of group A and B. The serum-worm mixtures were placed in Wassermann tubes and incubated at 37 C for 24 and 48 hours. At the end of each incubation period the serums were removed from the tubes by means of a capillary pipette and tested for their content of α_1, α_2 and β isoagglutinins. To the serum which was diluted 1:2, 1:4, etc., a drop of a 2% suspension of washed human erythrocytes of subgroups A_1 and A_2 and of group B was added. The mixtures in Wassermann tubes were placed in a water bath at 37 C for one hour and then examined microscopically for agglutinated erythrocytes at a magnification of ×20.

A bacteriological loopful from each bacterial culture was inoculated into 0.2 cc of human serums of groups A and B. The inoculated serums were incubated at 37 C for 24 hours. If growth was slight after 24 hours, the cultures were allowed to remain at 37 C for another 24 hours. After proper incubation the serum-bacteria mixtures were centrifuged at 10,000 r.p.m. for 15 minutes. The supernatant was removed with a capillary pipette and was then tested for α_1, α_2 and β isoagglutinins as was done with the serum separated from the worms.

A polysaccharide (glycogen) was prepared from the livers of normal rabbits by extracting with water and precipitation with alcohol. Twenty mg of this substance were added to 0.5 cc of serums of groups A and B. The serums thus treated were tested for blood agglutinins as with the serum treated with the helminths and with the bacteria.

Known numbers of trichina larvae were placed in serum from rabbits infected three months previously with this parasite, and the serum-worm mixtures were incubated at 37 C. Twenty-four hours later the serum was removed and tested for antipolysaccharide precipitins. For these tests the serum was placed in microprecipitin tubes layered with dilutions of purified polysaccharide antigen. The precipitin titer was recorded after one hour of incubation at 37 C.

Extraction of polysaccharide from worms and determinations of reducing sugar after hydrolysis with acid.—The worms left in the tube after the serum was removed from the suspension were digested in 20% potassium hydroxide in a boiling water bath for 20 minutes. An equal volume of 95% alcohol was then added, the mixture boiled again for a few seconds, and allowed to cool spontaneously. After cooling, the polysaccharide was allowed to settle overnight at 6 C. On the following morning the mixture was centrifuged and the supernatant discarded. The sediment was hydrolyzed in 5*N* sulfuric acid for 30 minutes and neutralized with sodium hydroxide, using phenol red as an indicator. To the neutralized solution appropriate amounts of 5% solution of zinc sulfate and 0.5% barium hydroxide were added.[6] The precipitate formed would remove other reducing substances coming from non-carbohydrate sources. After centrifugation, aliquot portions of the supernatant were used for the glucose determinations. Glucose was determined by the method of Somogyi,[6] using the Klett-Summerson photoelectric colorimeter. Similarly, the polysaccharide was also extracted from worms that had not been incubated in serum to serve as controls.

In several instances schistosome adults were placed in serum plus 0.075 volume of 1:15 *M* phosphate buffer (pH 7.4) and incubated 8, 16 and 24 hours at 37 C. The polysaccharide extraction and glucose determinations were performed as with the worms kept in serum with no phosphate.

Following the method of Melcher and Campbell,[7] a polysaccharide was also prepared by extracting ground trichina larvae in distilled water. The polysaccharide was hydrolyzed with 5 *N* acid and glucose determinations performed as with the polysaccharide obtained following extraction with potassium hydroxide. The amounts of reducing sugar obtained from the polysaccharide extracted with potassium hydroxide and water were thus compared.

RESULTS

Absorption of the α_2 isoagglutinin by substances present in live organisms.—All of the serum samples into which the worms or bacteria were incubated had a reduced α_1 and a negative α_2 isoagglutinin titer as compared with the titer of ther serums into which no organisms were introduced. The titers of the β isoagglutinin remained unchanged. Although there was some absorption of the α_1 agglutinin in the serums into which all of the infectious agents were inoculated, absorption was

6. Somogyi, M. 1948, J. Biol. Chem. **160**: 69.
7. Melcher, L. R. and Campbell, D. H. 1942, Science **96**: 266.

complete only in the case of the α_2 antibody (table 1).

The titer of the antipolysaccharide antibody in the serums from rabbits present in the hydrolyzed polysaccharide extracted from the trichina larvae and from the adults of *S. mansoni*, before and after incubation in human

TABLE 1.—*Isoagglutinin titers in terms of dilution of human serums of group A and B before and after incubation with the various infectious organisms.*

Number and species of organisms introduced into serum	Serum of group B* α_1 agglutinin		Serum of group B* α_2 agglutinin		Serum of group A* β agglutinin	
	Before incubation with organisms	After incubation with organisms	Before incubation with organisms	After incubation with organisms	Before incubation with organisms	After incubation with organisms
10,000 *T. spiralis* larvae	1,024	512	64	0	16	16
10,000 *T. spiralis* larvae	512	256	64	0	32	32
20 pairs of *S. mansoni*	128	64	128	0	32	32
20 pairs of *S. mansoni*	1,024	512	64	0	64	64
Streptococcus hemolyticus (B type)	512	256	64	0	32	32
Staphylococcus aureus	256	128	64	0	8	8
Staphylccoccus albus	512	128	128	0	16	16
Diplococcus pneumoniae type II	256	128	128	0	128	128
Corynebacterium xerose	256	32	32	0	8	8
Brucella abortus	128	32	32	0	64	64
Mycobacterium smegmatis	1,024	128	32	0	64	64
Bacterium typhosum	512	256	64	0	64	64
Shigella paradysenteriae type IV	512	128	128	0	128	128
Proteus OX19	1,024	128	32	0	16	16
Rabbit liver glycogen	256	256	32	32	32	32

* For the above tests the blood serum from 6 individuals of group B and the erythrocytes from 4 individuals of subgroup A_1 and 3 of subgroup A_2 were utilized.

TABLE 2.—*Absorption of antipolysaccharide antibody from serum of rabbits infected with* T. spiralis.

Number and species of organisms introduced into serum	Volume serum used	Precipitin titer against polysaccharide antigen	
		Before incubation with organisms	After incubation with organisms
40,000 *T. spiralis* larvae	1 cc	1:6,400	0
60,000 *T. spiralis* larvae	2 cc	1:12,800	0

TABLE 3.—*Polysaccharide determined as glucose in* T. spiralis *larvae and in adults of* S. mansoni *before and after incubation at 37 C in unheated human serum of group B and in serum of group B plus phosphate buffer.*

Number and species of worms utilized	Micrograms of glucose in worms						
	No incubation	Incubated 8 hours		Incubated 16 hours		Incubated 24 hours	
		Recovered	Percent lost	Recovered	Percent lost	Recovered	Percent lost
Unheated serum of group B							
10,000 *T. spiralis* larvae	411	216	47.4	128	68.8	69	83.2
10 adult pairs of *S. mansoni*	256					47	81.6
20 adult pairs of *S. mansoni*	552	164	70.3	120	78.3	51	90.8
20 *S. mansoni* males	431	271	37.1	245	45.5	76	82.3
Unheated serum of group B plus 0.075 volumes of 1/15*M* phosphate buffer (pH 7.4)							
20 *S. mansoni* males	253	140	44.6	40	84.2	0	100.0
20 *S. mansoni* males	466	198	61.9	144	69.1	20	95.7

infected with *T. spiralis* was reduced to zero when the serum was incubated with larvae of this parasite (table 2).

Reduction of the polysaccharide content of the worm as a result of incubation in serum.—The determinations of glucose serums of group B, are presented in table 3. In all cases the glucose determinations done on the worms incubated for 24 hours were at least 80% less than the values obtained previous to incubation.

When the serum-phosphate mixture was used, the worms lost their polysaccharide more rapidly than in the serum with no phosphate added.

There was no significant difference between the amounts of polysaccharide extracted from trichina larvae using either water or potassium hydroxide (table 4).

TABLE 4.—*Polysaccharide determined as glucose in* T. spiralis *larvae after extraction with potassium hydroxide and after extraction with water.*

Number of larvae utilized	Micrograms of polysaccharide in terms of glucose	
	Extracted with potassium hydroxide	Extracted with water
10,000	471	448
10,000	510	476
20,000	832	844

DISCUSSION

The complete absorption of the α_2 agglutinin in the serum of group B by substances present in all of the live organisms tested is a matter of great interest. Previously, such substances with isoagglutinogen-like properties had been isolated by means of chemical treatment of the organisms concerned but had not been found as a metabolite produced by the live organism. Although there is a reduction in the titer of the α_1 agglutinin, this may not be significant. Absorption of an isoagglutinin by the homologous isoagglutinogen often causes a diminution in titer of the heterologous antibody due to a non-specific absorption or perhaps, more so, to the fact that the two agglutinins may be partly bound together.[8] The fact that the titer of the β agglutinin in serum of group A remained unchanged and that the titer of the α_1 was only slightly reduced indicates that the absorption of the α_2 agglutinin is not merely due to destruction of the antibody proteins by growing bacteria, since in such a case all antibodies would have disappeared. When glycogen prepared from a rabbit liver was added to serums of groups A and B, the titers of the α_1, α_2 and β agglutinin remained unaltered. This strengthens the probability that the absorption of the α_2 agglutinin by the bacterial substances is of a specific nature and is not dependent on non-specific factors.

The findings of this study suggest that the absorption of the α_2 agglutinin is a natural phenomenon taking place whenever live organisms come in contact with blood serum. This may be the case when organisms invade the blood of their host, so that a vital function of the normal isoagglutinins in blood is, perhaps, to neutralize or destroy such metabolic products coming from the invaders.

The property of absorbing the α_2 isoagglutinin as possessed by the organisms studied necessarily postulates a functional antigen common to all of them. Therefore, cross reactions would occur among all infectious organisms. Although no reports have been made as to the findings of cross reactions between such distant groups as helminths and bacteria, these may have been overlooked. Following treatment of the infectious organisms with strong acid or alkali, the polysaccharide extracted loses its property of inhibiting the isoagglutinins in human serum.[9] This fact may also partly explain why such cross reactions have been overlooked.

If infectious organisms possess an A_2 agglutinogen, as suggested by the findings in the above experiments, the following facts should be considered. Upon infection with the organisms, α_2 agglutinin may develop in the host; and if the host has the homologous aggluti-

8. Landsteiner, K. and Witt, H. D. 1926, J. Immunol. **11**: 221.

9. Oliver-González, J. 1946, J. Infect. Dis. **79**: 221.

nogen in its erythrocytes, autoagglutination may develop.[9] The α_2 agglutinin (also called anti-O) is not specific for A_2 cells, but also gives weak reactions with certain bloods of group A_1 and B. Therefore, autoagglutination may occur in all blood cells which react with α_2 agglutinin.

The antigenic substance present in all blood groups which react with α_2 or the anti-O agglutinin is inferred as being hereditary. This agglutinin behaves like agglutinins present in some eel serum, which acts strongly on group O cells and which separates A, B and AB cells into two types according to degree of agglutination,[10]—one type in which A, B and AB cells are agglutinated by highly diluted or highly titered serum (type E), and a second type in which these cells are agglutinated by low titered eel serum (type e). The agglutinogens E and e in eel blood are hereditary qualities. Thus, from this it may be inferred that the antigenic substances in all human red blood cells which react with anti-O or α_2 agglutinin are also hereditary and that human bloods may be divided into two groups according to the presence or absence of such a substance. It may be further inferred that if autoagglutination of erythrocytes occurs as a result of infection with organisms, it may be related to the presence of the A_2 substance in the cells.

When trichina larvae were incubated in serum from infected rabbits, the antipolysaccharide antibody was absorbed from the serum. This indicates that the substance as secreted into the serum by the organisms is the immunologically active polysaccharide and not a degradation product of the polysaccharide, such as free glucose.

The polysaccharide which is lost by the worm may not necessarily be the same substance which absorbs the isoagglutinins from the serum. However, there are certain facts which suggest that such is the case. The polysaccharide which is obtained from trichina larvae by treatment with potassium hydroxide is obtained in about the same amount as the polysaccharide obtained by water extraction. Although no inhibition of the agglutinins is obtained with potassium hydroxide-extracted material, the agglutinins are inhibited with water-extracted material. Therefore, a reduction in the one substance probably signifies a reduction in the other.

Absorption of the α_2 isoagglutinin may be performed by a small fraction of the polysaccharide-complex substance in the worm, which has not been metabolized completely by enzymes in the worm or in the host. The presence of large amounts of non-metabolized polysaccharide may be harmful to the host. This may be the case during massive invasions by infectious agents in which the host may not be able to dispose of the polysaccharide rapidly enough.

The release of the polysaccharide by the infective organism upon its penetration into the host may be essential for its survival. The introduction of poisons or inhibitors into the enzyme systems which act on the splitting of the carbohydrate from the rest of the complex may be more harmful to the organism than inhibitors introduced at the beginning or near the end stages of glycolysis.

SUMMARY AND CONCLUSIONS

1. When known number of *Trichinella spiralis* larvae and adults of *Schistosoma mansoni* were incubated in human serum of group B, complete absorption of the α_2 isoagglutinin in the serum was observed. The titer of this agglutinin, as tested with homologous group A_2 erythrocytes, was reduced to zero. Also, the titer of the α_1 isoagglutinin was

10. Sugishita, G. 1935, Juzenkai-Zasshi (Japanese) **40**: 336.

reduced as compared with the titer of the untreated serum. The reduction in the titer of the α_1 agglutinin, however, may have been due to the fact that the α_1 and α_2 agglutinins are partly bound together.

2. The titer of the β isoagglutinin in human serums of group A was not altered when the organisms were introduced into the serum.

3. Similarly, when bacteria, representing various groups and belonging to 10 species, were introduced into serum of group B, complete absorption of the α_2 and a reduction of the α_1 isoagglutinins was also observed. The specific absorption of the α_2 agglutinin and the non-absorbability of the α_1 and β from serums of groups B and A indicates that the absorption is not due to destruction of antibody proteins by growing bacteria but to a specific absorption by the agglutinogen present in the secretions of the organisms.

4. The absorption of the α_2 isoagglutinin by the live organisms suggests the presence of a common antigen among them. This common antigen may be involved in cross reactions which may take place among the various infectious agents.

5. When the larvae of *T. spiralis* and adults of *S. mansoni* were incubated for 8, 16 and 24 hours in human serum of group B, a reduction of the polysaccharide content of the worm was observed. The polysaccharide was determined by the amount of reducing sugar after hydrolysis with acid.

6. All evidence seems to indicate that the polysaccharide lost by the larvae of *T. spiralis* and adults of *S. mansoni*, when incubated in serum, is the same substance which absorbs the α_2 agglutinin.

7. The substance which is released into the serum by the organisms is probably the intact polysaccharide and not a digestion product. This is suggested by the fact that this substance as secreted by larvae of *T. spiralis* absorbs the antipolysaccharide antibody in serum from infected animals.

8. The significance of the presence of the isoagglutinogen-like substance in infectious organisms is discussed.

NOCARDIOSIS, WITH A REPORT OF THREE CASES OF ACTINOMYCOSIS DUE TO *NOCARDIA ASTEROIDES*

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A survey of the literature reveals that infection due to *Nocardia asteroides* (formerly called *Actinomyces asteroides*[1]) is not common. The review by Henrici and Gardner[2] in 1921 contained only 26 reports of infection with these aerobic acid-fast actinomyces. All but three were apparently pulmonary infections, two were postoperative peritonitis and one was Madura foot. All died except the one with Madura foot, which was cured by amputation. The causative organisms fell into 4 different types or strains:

1. Eppinger's strain, or *Nocardia asteroides*. This strain produced a growth of mealy consistency on solid medium, varying from pale yellow to deep orange in color. It did not liquefy gelatin nor peptonize milk, and was more pathogenic for guinea pigs than for rabbits.

2. Aoyama and Miyamoto's strain. This strain differed from the *N. asteroides* only in the white color of the colony growth and the darkening of the culture medium.

3. Birt and Leishman's strain. This strain produced a snow-white growth on a solid medium. It did not liquefy gelatin but did peptonize milk and was pathogenic for guinea pigs.

4. Berestneff's strain. This strain liquified gelatin and was not pathogenic for laboratory animals.

In addition to these 4 strains, Henrici and Gardner added a 5th strain, for which they proposed the name *Actinomyces gypsoides* because of the chalky-white growth on solid culture mediums. This strain had proteolytic activity as evidenced by the alkaline coagulation of milk, the rapid liquefaction of gelatin, and the darkening of mediums containing peptone. However, this organism gradually lost its proteolytic activity, first failing to peptonize milk, and then to liquefy gelatin, and could be differentiated from *N. asteroides* only by the brown color on agar and a more pronounced tendency to form aerial mycelium. Later Henrici[3] expressed the opinion that the species *N. asteroides* varied through a wide range and that if species were established on the basis of small differences in biological characters, there would be about as many species as strains of organisms isolated. He was inclined to agree with Baldacci,[4] who proposed reducing all *N. gypsoides* and relative strains of the aerobic acid-fast actinomyces with only minor differences in their biological characteristics to the rank of variants of *N. asteroides*.

In their review of the literature, Henrici and Gardner did not include a number of infections due to this group of organisms.[5,6,7,8,9] Since 1921 a number

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4. Baldacci, E. 1938, Revisione di alcune specie del. g. Actinomyces, Mycopathologia. **1**: 68.
5. Ohlmacher, A. P. 1902, An atypical acid- and alcohol-proof fungus from the sputum of a case resembling pulmonary tuberculosis. Cleveland Med. J. **1**: 29.
6. Steele, A. E. and Lee, R. I. 1913, A case of in-

of additional infections apparently by aerobic, variably acid-fast actinomyces have been reported, some of which, for various reasons, have not been considered acceptable,[10,11,12,13,14] but about 10 of which have been considered acceptable by subsequent authors for inclusion among the diseases caused by *N. asteroides*. In 1937 Goldsworthy[15] reported pulmonary actinomycosis due to an aerobic acid-fast species in an Australian male, 69 years of age, which terminated fatally. In 1938 Kessel and Goolden[16] reported a comparison of strains of actinomyces recovered from human lesions. Included was one fatal infection, where cultures taken from the lungs and meninges yielded an acid-fast aerobic actinomyces. About this time sulfonamide therapy was introduced. In 1944 Benbow, Smith and Grimson[17] described two patients, one a farmer's wife and another a farmer, infected with *N. asteroides*, both of whom recovered from the pulmonary infection under rest, surgery, vitamins, sulfonamides and iodides. In 1945 Binford and Lane[18] reported a fatal infection of chronic suppurative pneumonitis, massive brain abscess, and bilateral ischiorectal abscesses from which a fungus identified as *N. asteroides* was obtained. In 1946 Kirby and McNaught[19] reported two patients with *N. asteroides* infection. The first was a man aged 63 years with productive cough, loss of weight and pulmonary disease. He responded well to sulfadiazine therapy, but the etiologic agent was not recognized until late in the disease, and subsequently he died. The other patient was a women aged 49 years who received hospital care for 5 days prior to death with a clinical diagnosis of an intracranial lesion without localizing symptoms. The necropsy revealed an abscess in the cerebellum from which *N. asteroides* was cultured. Shaw, Holt and Ray[20] in 1946 reported the illness of a white woman aged 32 years suffering from a pulmonary infection simulating tuberculosis. The diagnosis was established by bacteriologic study of the pleural fluid. The

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patient recovered following surgical drainage of an empyema cavity and of an abscess in the right buttock, along with penicillin, potassium iodide, thymol and sulfadiazine therapy. In the same year Calero[21] reported an infection of a white girl aged 12 years suffering from "pleuro-pulmonary fistulae." *N. asteroides* was isolated from the fluid obtained from the fistulae and from the sputum. She received several courses of sulfonamide drugs and two courses of penicillin. She was dismissed as cured although the sedimentation rate remained eleveated. Calero pointed out that the final outcome could not be given at the time the report was written. Glover, Herrell, Heilman and Pfuetze[22] reported *N. asteroides* infection in a white male physician age 26 years in whom all studies failed to confirm the diagnosis of tuberculosis. On the other hand, cultures of the empyema fluid yielded *N. asteroides*. Penicillin and streptomycin therapy had no effect on the course of the infection. Intensive sulfadiazine therapy was followed by apparent complete recovery.

Since 1938 many reports indicate the effectiveness of the sulfonamide compounds and antibiotics in the treatment of infection with *Actinomyces bovis*.[23,24,25,26] From studies carried out by Glover, Herrell, Heilman and Pfuetze and those carried out elsewhere, sulfadiazine is the drug of choice in the treatment of actinomycosis due to *N. asteroides*. Other studies[22,27,28] cast a doubt on the sensitivity of *N. asteroides* to penicillin. The same might be said of streptomycin, but a true evaluation of these and other antibiotics must await further experimental and clinical studies.

After exclusion of the patient with Madura foot cured by amputation and the case reported by Henrici and Gardner whose final outcome was not given, only 5 of the 37 cases cited from the literature appear to have recovered. All 5 patients received sulfonamide therapy in addition to other forms of treatment. Three received penicillin and one streptomycin. In 21 of the 37 cases the diagnosis was made only at necropsy, and the organism was demonstrated in 16 during life, usually in the terminal stage of the disease. The brain had metastases in at least 11 cases.

Since aerobic types of actinomyces may be highly pathogenic to man and are not as well known as the anaerobic *A. bovis*, three patients infected with an aerobic variety are described, not only because of their rarity, but also to emphasize the necessity of considering mycotic disease, particularly nocardiosis in the differential diagnosis of obscure infections, and to outline some of the procedures necessary to establish a diagnosis.

Case No. 1

Clinical brief.—J. H., a white male laborer aged 39 years, was admitted to the service of

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Dr. W. R. Cubbins at St. Luke's Hospital on October 16, 1941, complaining of a suppurating wound of the right thigh following a traumatic contusion 6½ months previously, headaches for three months, clonic seizures of the right arm for 6 weeks, blurred vision for five weeks, difficulty in walking, aphasia and slurred speech for two weeks, and incontinence for several days. The large suppurating wound on the upper right thigh had many sinuses which burrowed beneath the subcutaneous tissues and communicated with one another. Neurological findings pointed to a left cerebral abscess. There was a slight anemia and leucocytosis of the blood, but other laboratory procedures were within normal limits. Three days after admission a left subtemporal exposure of the brain revealed deep in the occipital lobe posteriorly a hard mass from which 15 cc of an odorless, greyish-yellow viscid fluid were aspirated. He failed to rally after the operation, and died on October 20, 1941, 7 months after his initial injury.

Necropsy.—Only pertinent portions of the necropsy are mentioned. Six open granulating wounds were in the upper two-thirds of the right thigh along the medial surface, lying along the adductor muscles and connected with each other under the separating bridges of skin and subcutaneous tissue. There was a recent surgical left subtemporal decompression wound of the scalp and calvarium. After the brain was hardened in formalin, it was cut into coronal sections ranging to 2 cm in thickness. A greenish grey abscess 3 by 4 by 4 cm was in the left temporal lobe. The central portion was soft and the wall was dense fibrous tissue. Another similar abscess in the frontal lobe of the right cerebral hemisphere was 1.2 by 1 by 1 cm. The center of the two abscesses histologically had necrotic cellular debris with a vague fibrillar structure. The wall of the abscesses was a narrow margin of granulation tissue with many fibroblasts, glia cells, dilated capillaries, and external to this was a wider zone of glial proliferation.

Bacteriology and mycology.—The exudates from the abscess cavity in the left occipital lobe and from the region of cellulitis of the right thigh grew out on blood agar plates at the end of 48 hours into several flat, dry, grey-white colonies 0.5 mm in diameter, which were discrete and produced no change in the surrounding blood agar. Similar growths were on the semianaerobic slants. At the end of 96 hours the colonies were 1 mm in diameter, chalky-white, slightly raised and with a central ring. They adhered to the agar but emulsified fairly easily. Stained preparations disclosed a fine tangled mycelial mass in which the central filaments were closely intertwined, the peripheral portions being arranged irregularly in a radial fashion. The mycelium was not uniform in its acid-fastness. After a week the colonies became yellow, the color then deepened to orange, and then to coral red. The same characteristics were observed with nutrient agar on which the growth was fairly luxuriant. In broth the organism formed fluffy grey-white separate particles from 0.2 to 0.5 cm. After 8 days a surface pellicle of dry, separate chalky particles averaging 1 mm in diameter formed. These became yellow with age. No fermentation after two weeks occurred in cultures containing dextrose, mannite, maltose, lactose, salicin, sucrose, xylose, levulose, sorbitol, trehalose, galactose and arabinose. No fermentation of dextrin or splitting of peptone occurred although growth was abundant. Gelatin was not liquefied. In agar stabs, growth occurred at the base along the stab line and on the surface of the agar. In most preparations, including slide cultures and smears stained with Gram's, methylene blue and Ziehl-Neelsen's stains, the organisms appeared as interlacing, slender, branching, fragmenting, non-sporulating, gram-positive variably acid-fast filaments. The average width of the filaments was about 1 μ. The colonies had a tangled, fairly dense central mycelial mat from which filaments radiated in all directions. The

filaments varied in length, but in the oldest colonies as well as those growing most actively, they never were longer than 40 μ. Those in the young colonies were almost straight, in older colonies spiral, becoming more spiral as the colony aged. The filaments branched along their course, lateral branches coming off usually at right angles but sometimes at obtuse angles to the main filament. The branches were short, averaging 7 to 9 μ. In older colonies these appeared to anastomose.

Pure cultures 6 weeks old in plain broth were used for the animal tests. The results of animal inoculations with broth cultures of the fungus were as follows: Rats injected intraperitoneally with 2 cc of broth culture were dead or moribund in 5 to 6 days. Caseous subcutaneous abscesses at the site of injection, peritoneal nodules, omental nodules, the spleen and the kidneys yielded growths similar to those described. One large male rat injected intraperitoneally with 2 cc of a 4-week broth culture was killed after 6 days, and an intense inflammation of both testes was found, as well as a nodular peritonitis. A guinea pig inoculated intraperitoneally with 2 cc of a 6-week broth culture was killed in 4 weeks. There was no peritoneal exudate. Cultures of the organs were negative, except the liver, from which one colony was obtained. A white mouse inoculated intraperitoneally with 0.5 cc of a 6-week broth culture was killed on the 14th day. Cultures from the spleen had one colony of the fungus. A white rabbit was inoculated intravenously with 2 cc of a 6-week broth culture and 4 days later with 2 cc intraperitoneally. The rabbit was found dead on the 5th day. Cultures of the liver were positive. Another white rabbit inoculated with 2 cc directly into the basal cistern was killed after three weeks. No changes were present and cultures were negative. Histological studies of the tissues removed from these animals had necrotic regions with changes similar to those found in the brain of the patient, in the kidney of the first rabbit, in the subcutaneous tissue at the site of injection, in the liver and omental fat of one rat, in the lung and pancreas of the mouse and in the lung and spleen of the guinea pig.

The significant anatomical, histological and bacteriological diagnoses of the patient are summarized as follows: chronic cellulitis and multiple fistulae (*N. asteroides*) of the skin and subcutaneous tissues of the right thigh; embolic abscesses (2) of the cerebral hemispheres of the brain.

Case No. 2

Clinical brief.—R. S., a white male farmer aged 30 years, was admitted to the service of Dr. N. C. Gilbert at St. Luke's Hospital for the third time on March 8, 1947. His first admission to St. Luke's Hospital was on August 19, 1946, because of a nose and throat infection of 5 weeks' duration. The patient had a sore throat, fever and an enlarged right submaxillary lymph node which broke down after one week and drained into his throat. One week after the onset of the sore throat he had a large granuloma in the left nostril which bled freely. The left side of his face became red and swollen, viscid yellow exudate drained from the inner canthus of the left eye, and he had left-sided headaches. Because he did not respond to penicillin, sulfadiazine and hot packs locally, he was referred to St. Luke's hospital by his physician. He was treated with penicillin, streptomycin and sulfadiazine. He continued to have fever ranging between 101 and 103 F. He was discharged October 3, 1946, improved but undiagnosed. About December 1, 1946, he noticed a profuse purulent drainage from the sinus in the floor of the mouth and increasingly tender right cervical nodes. He was readmitted to the hospital on December 16, 1946. A pleural friction rub was over the right chest, and there were diminished tactile fremitus and breath sounds over the base of the right lung. Cultures from the nose and throat had *Streptococcus hemolyticus*, *Staphylococcus aureus* and *Neisseria catarrhalis*. There were 2,480,000 erythrocytes and 9550 leucocytes per cmm of blood. He received several blood transfusions. A lymph node removed from the left axilla had chronic inflammation. He was only slightly im-

proved when discharged on January 14, 1947, and was readmitted for the last time on March 8, 1947, with bilateral bronchopneumonia, a fever of 103.8 F, cough, hemoptysis and had lost 30 pounds in weight. He had received 800 r units of roentgen-ray therapy over the nose and left antrum from February 19 to 22, 1947. The blood contained 1,940,000 erythrocytes and 12,000 leucocytes per cmm, and 4.8 gm percent of hemoglobin. The urine had a specific gravity of 1.014 and contained 100 mg percent of albumin. The patient was given penicillin and intravenous fluids, but continued to decline and died on March 10, 1947, about 8 months after the onset of his illness.

Necropsy.—The axillary and mesenteric lymph nodes were moderately enlarged and histologically had small focal regions of necrosis surrounded by a dense zone of polymorphonuclear leucocytes, many of them eosinophils. In these foci were faint filamentous structures less than 1 μ in diameter. The lining of the trachea and bronchi was hyperemic and covered with thin deposits of fibrin. In the lining of the left bronchus and trachea were multiple superficial ulcers. Histologically the mucosa had fibroplastic tissues with exudates of polymorphonuclear leucocytes, lymphocytes, plasma cells, mononuclear phagocytes containing brown granular pigment, and many multinucleated giant cells. These exudates extended through the muscularis mucosa into the submucosa, muscularis and adventitia (see fig. 1). The right lung weighed 1200 and the left 1410 g. They were dark red mottled with carbon pigment, acrepitant and firm. The histological structure of the lungs varied considerably. At some levels there was an acute hemorrhagic inflammation (see fig. 2) with extravasations of red blood cells, polymorphonuclear leucocytes, many eosinophilic, and mononuclear phagocytes. At other levels the process was a subacute bronchopneumonia (see fig. 3), whereas at still other levels the process was a chronic organizing bronchopneumonia (see fig. 4). In foci of necrosis were filamentous structures which appeared to be gram-positive and variably acid-fast. Thrombosis with partial organization of the blood vessels also occurred (see fig. 5). Such thrombi had narrow filamentous structures less than 1 μ in diameter suggestive of fungus hyphae (see fig. 6). The spleen weighed 380 g. The red pulp was moderately engorged with red blood cells and the Malpighian bodies were prominent. Scattered widely were focal regions of necrosis with exudates of polymorphonuclear leucotyes (see fig. 7) containing narrow filamentous structures similar to those described. Some of these foci of necrosis were partially organized (see fig. 8). The lining of the small and large bowel had many scattered oval ulcers. In these, portions of the mucosa were necrotic and contained filamentous structures suggestive of fungus hyphae.

Bacteriology and mycology.—Cultures of the lungs, the peritoneal fluid, the pleural fluid and the pericardial fluid on glucose agar yielded colonies of a fungus which appeared as thin greyish veils, in two or three days becoming thick, opaque and chalky-white in color, presumably from the aerial mycelia, the surface being dry and wrinkled. The growth adhered firmly to the medium, and when the whitish bloom was scraped away the underlying mycelium had a buff color. The fungus had a pungent, musty odor at first, which gradually grew less intense after several months. In glucose broth the fungus grew as thread-like filaments at the bottom of the medium. In slide cultures and in slide preparations stained with Gram's stain and methylene blue, the organisms appeared as interlacing, slender branching, fragmenting, non-sporulating gram-positive filaments variably acid-fast, depending on the strength of the decolorizing agent, the length of time placed in the decolorizing

agent, and the filamentous, bacillary or coccoid forms present. No fermentation occurred with luxuriant growth in broth mediums containing dextrose, lactose, sucrose, maltose, mannite, salicin, trehalose, sorbitol, arabinose, levulose, galactose, dextrin, xylose, raffinose and inulin. Gelatin was liquefied and litmus milk was peptonized with the formation of an alkaline solution. Peptone water was discolored brown. After a few weeks the subcultures had a less pungent musty odor, failed to peptonize milk, liquefied gelatin more slowly and discolored peptone water less intensely than earlier subcultures. The organism failed to grow anaerobically on Sabouraud's dextrose agar.

Two mice, 2 rats and 4 guinea pigs were injected intraperitoneally and 6 rabbits intravenously with saline suspensions of colonies scraped from glucose agar subculture, except the guinea pigs and 2 rabbits, which were inoculated with sediment of glucose broth subcultures. Of these animals, only one rabbit inoculated intravenously with a subculture of the fungus obtained from the pleural fluid had significant pathological findings. Sacrificed after three weeks, one lobe of the liver had multiple abscesses with central masses of basophilic-staining material and radiating peripheral lobulated structures like "clubs," surrounded by a zone of fibroplastic tissue containing many lymphocytes, plasma cells, polymorphonuclear leucocytes, and some eosinophil and mononuclear phagocytes. These lesions were similar to some described by Henrici[3] in animals when the virulence of the organism is low. A culture of the pericardial fluid of this rabbit yielded a single colony of a chalky-white fungus from which subcultures were grown that had the same characteristics as subcultures of the fungus obtained from the patient. The organism recovered from the rabbit was able to peptonize litmus milk, liquefy gelatin, and discolor peptone water, properties lost by subcultures of the fungus obtained from the patient.

As most mycologists will agree, no group of micro-organisms presents so much difficulty in classification as that now generally designated *Actinomycetes*, in which group this organism falls. Because of the mycelial growth, animal pathogenicity, cultural and staining characteristics, it was thought that this organism was identical or closely related to the strain Henrici named *Actinomyces gypsoides*, and later renamed *Nocardia asteroides*. Because of the extreme variability of the actinomycetes in both morphological and cultural characteristics, it was thought advisable to send cultures of the organism to several well-known mycologists for verification of the identification of the organism. Principle Mycologist C. W. Emmons of the National Institute of Health at Bethesda, Maryland, thought the fungus bore some resemblance to *N. asteroides*, but that it was not entirely typical. He was not able to produce disease in animals, although he was able to recover the fungus by culture if the animal was killed soon after the experimental inoculation. Dr. Norman F. Conant of the Department of Bacteriology at the School of Medicine, Duke University, thought the fungus was a *Nocardia* species, and that *N. gypsoides* was as close an identification as could be made. Dr. E. D. DeLamater of the Section on Bacteriology at the Mayo Clinic, Rochester, Minnesota, thought that on preliminary observation the organism was a "so-called gypsoides."

The anatomical, histological and bacteriological diagnoses are summarized as follows: extensive confluent acute exudative and chronic organizing bronchopneumonia of both lungs (*N. asteroides*); marked ulcerative tracheitis and

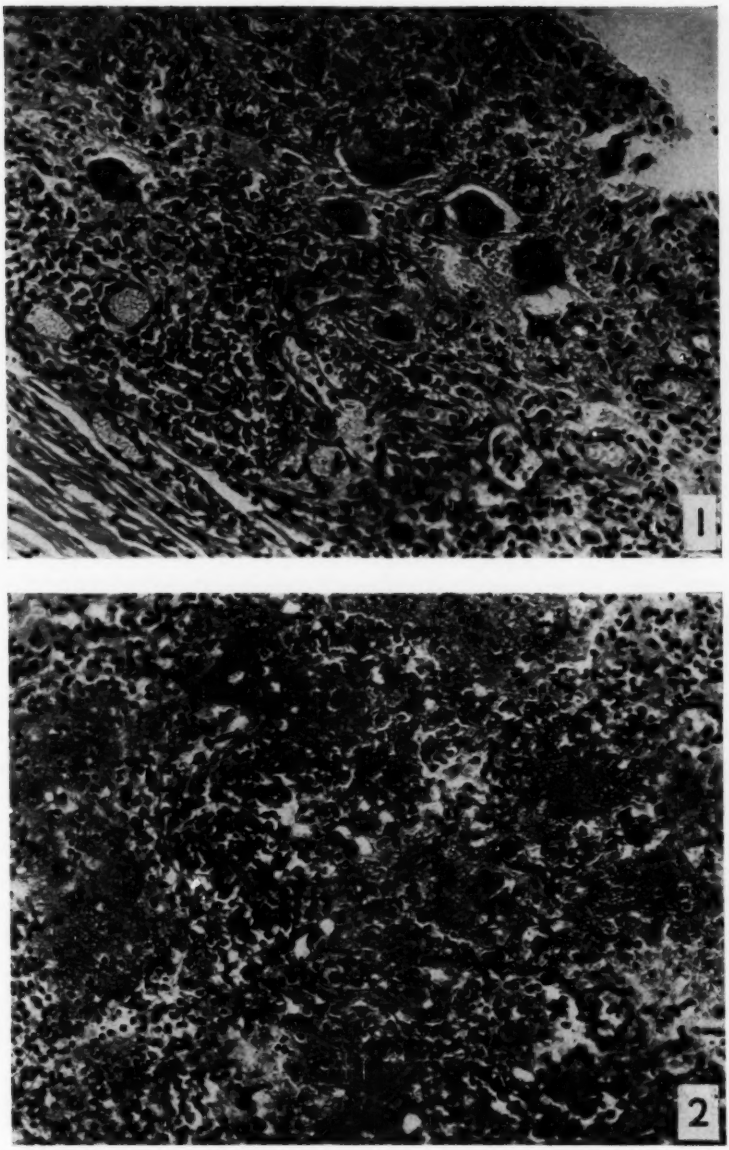


FIG. 1.—Photomicrograph illustrating the marked ulcerative and inflammatory changes of the lining and wall of the trachea. Note the multinucleated giant cells. ×198.
FIG. 2.—Photomicrograph illustrating the acute hemorrhagic bronchopneumonia of the lungs. ×198.

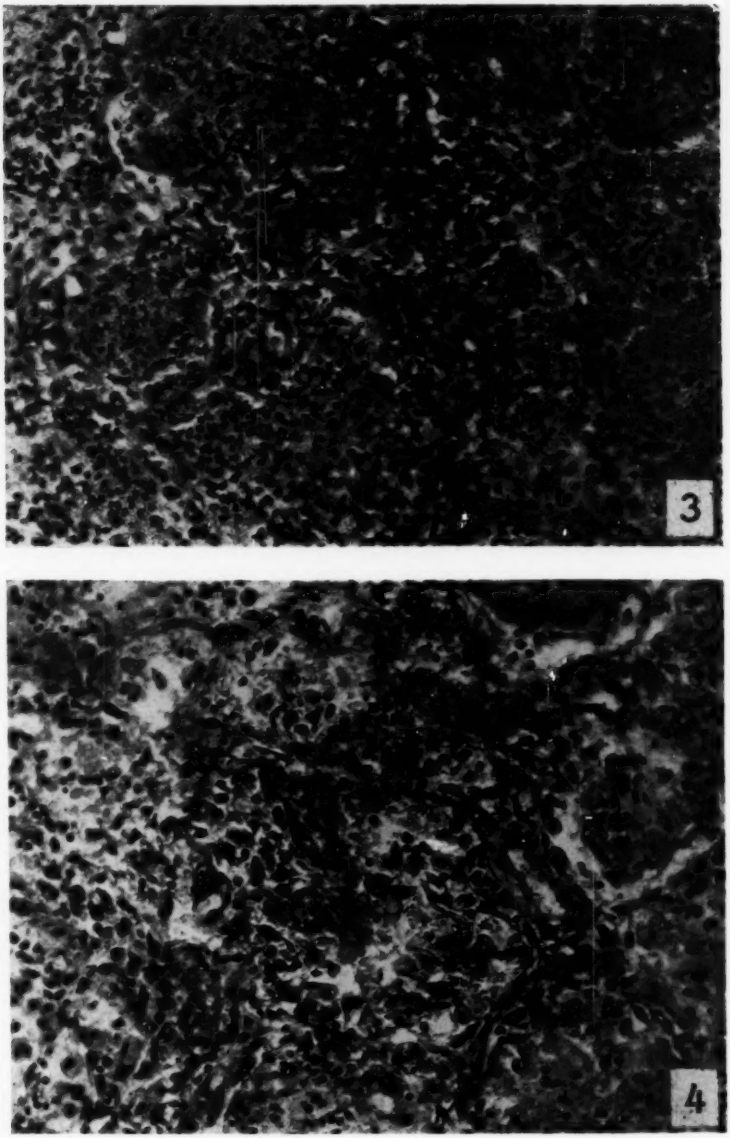
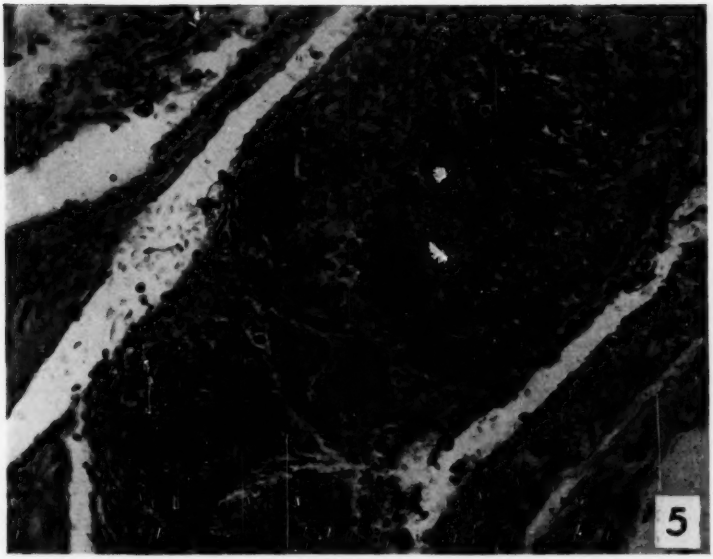


FIG. 3.—Photomicrograph illustrating the subacute bronchopneumonic process in the lungs. ×198.
FIG. 4.—Photomicrograph illustrating the organizing bronchopneumonic process in the lungs. ×198.



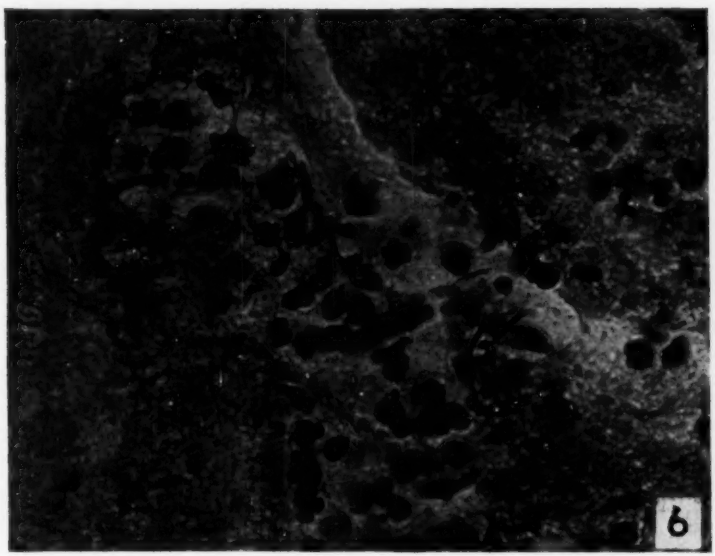
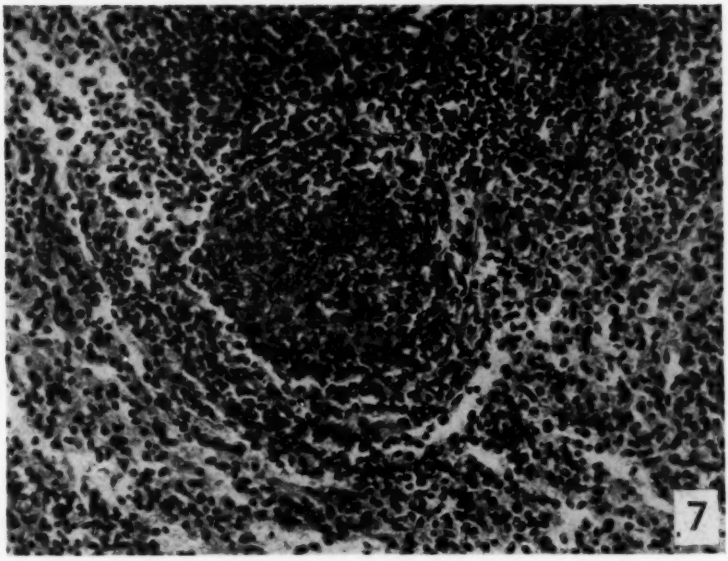


FIG. 5.—Photomicrograph illustrating a partially organized thrombosed blood vessel in the lung. ×198.

FIG. 6.—High power view of the thrombus illustrating filamentous structures.



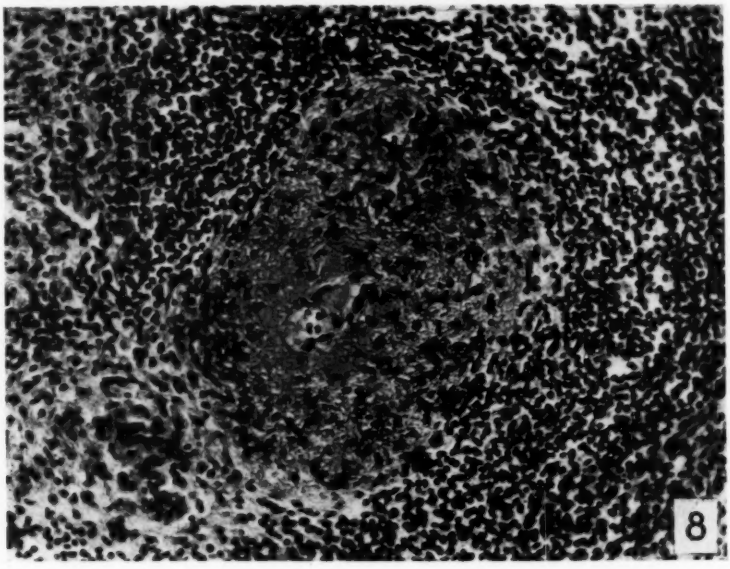


FIG. 7.—Photomicrograph illustrating a focus of acute inflammation of the spleen with a central region of necrosis. ×198.

FIG. 8.—Photomicrograph illustrating a region of inflammation with organization in the spleen. ×198.

bronchitis; focal acute and chronic organizing inflammation and hyperemia of the spleen; focal acute and chronic organizing inflammation and hyperplasia of the lymph nodes; and acute ulcerative inflammation of the lining of the small and large bowel.

Case No. 3

Clinical brief.—A single white female aged 23 years was admitted to the service of Dr. G. W. Scupham at St. Luke's Hospital on January 28, 1948 for two months and again on October 5, 1948 for two weeks. She entered the hospital the first time because of a mass of 4 weeks' duration under the right mandible. Four months before admission the right lower wisdom tooth was extracted without complications. A month before admission she noticed tenderness along the angle of the right mandible which localized in a mass under the right mandible. During the week before admission this mass increased in size. Laboratory studies were within normal limits, except for a slight leucocytosis. A tuberculin skin test was negative. Despite penicillin therapy (30,000 units every 3 hours for 6 days), the mass under the right mandible became more tender, and on the 12th hospital day this was incised and drained. Stained preparations of the exudate had no bacteria, but in cultures, colonies of *N. asteroides* appeared. She was given streptomycin. Blood cultures and roentgenograms of the lungs were negative. On February 26, the 30th hospital day, the mass was drained again. Stained preparations of the exudate revealed no bacteria, but in cultures a few colonies of *N. asteroides* appeared. The indurated mass at the angle of the jaw was opened and drained on March 4, and again the exudate in cultures had colonies of *N. asteroides*. She was given sulfadizine, sodium bicarbonate and potassium iodide. Subsequent cultures of the draining wounds had a few colonies of *Staph. aureus* but no *N. asteroides*. After 65 days in the hospital, she was discharged with two wounds draining slightly. She continued the sulfadiazine and sodium bicarbonate therapy for 2½ months, with a gradual healing of the wounds of the neck after 1½ months. She regained her normal weight and resumed her normal activities. However, on September 28, about 6 months later, she noticed a small lump in the right submandibular region just posterior to the previous sites of infection. On October 5, 1948 she again entered the hospital and on October 13 the mass was drained. The chronic granulation tissues removed had irregular thread-like masses. After two weeks in the hospital, the wound seemed to be healing and she was discharged. At the time of this report the outcome is not known, but the morphological and cultural characteristics of the organism isolated were those of *N. asteroides*.

COMMENT

Geographically the distribution of the aerobic type of actinomycosis is worldwide, cases having been reported from America, England, France, Germany, South Africa, Japan, Australia and the Philippine Islands. In 1925 Sanford and Voelker[29] reviewed the incidence of actinomycosis in the United States, and up to that time, of 670 cases reviewed, about 10 were due to the acid-fast type of actinomyces. The ages of the patients with the acid-fast type of actinomycosis extend from 4 weeks to 70 years, and the disease appears to be fairly well distributed through adult life, mostly in males. Actinomycosis due to the anaerobic actinomycetes is usually considered as an occupational disease, the majority of the cases occurring in men who handle grain or straw or similar material on which the organisms are believed to live. With the acid-fast actinomycetes, however, no such clear-cut occupational hazard can be cited, for only a few of the reported patients were grain handlers. In the vast majority of the reported cases the primary infection was in the respiratory tract, and was accompanied frequently by empyema or by metastases, the latter localizing most frequently in the brain. The duration of the illness varied from two weeks to three years, with about 6 months as the average. Microscopically, the lesions in human tissues are foci of necrosis with exudate cells in the vicinity of the mycelium. These regions are surrounded by granulation tissue containing mononuclear phagocytes and gradually become scar tissues. The le-

29. Sanford, A. H. and Voelker, M. 1925, Actinomycosis in the United States. Arch. Surg. **11**: 809.

sions in experimental animals are similar, except when the virulence of the organism is attenuated, lesions simulating those produced by *A. bovis* may be formed. The prognosis until the advent of the sulfonamides was poor, but in recent years has improved considerably.

As Benbow, Smith and Grimson[17] have stated, the fungi causing actinomycosis in man belong to two biologic types. The anaerobic type, *A. bovis*, is gram-positive and non-acid-fast and is found in approximately 90% of the cases of clinical actinomycosis, usually referred to as the "lumpy jaw" or the ordinary type of actinomycosis. It usually produces sulfur granules in the tissues, composed of mycelial masses with "radial club" arrangement at the periphery. This "club" formation is such a classic part of the description of this organism that in its absence the detection of actinomyces may be confused. The aerobic type includes several species, all of which are gram-positive and some of which are partially or totally acid-fast. The latter may be distinguished from the organisms of "lumpy-jaw" or ordinary actinomycosis in man because they do not usually form clubs at the extremity of the mycelium in infected tissues, human or other animal. The aerobic actinomyces may be divided into two groups,—a larger group which has no tendency to retain the stain when stained by the usual method for demonstrating acid-fast organisms, and a much smaller group composed of organisms which are partially acid-fast but are never as resistant to decolorization as the tubercle bacilli. The aerobic *N. asteroides* is of this type and has been studied the most.

The aerobic type of organism, irrespective of the acid-fast properties, is frequently described in the English and American literature as *Streptothrix* and in the French and South American literature as *Nocardia*. In a recent article on the nomenclature and classification of the actinomycetes, Henrici and Waksman concluded that the valid name for the aerobic, fragmenting, nonsporulating types of actinomycetes is *Nocardia*.[1,30] Glover, Herrell, Heilman and Pfuetze[22] suggested the term "actinomycosis" be restricted to infection due to *A. bovis* and the term "nocardiosis" be applied to infection due to *N. asteroides*.

The actinomycetes are an important group of fungi, but on the whole, the usual medical textbooks treat the subject casually. The earliest records are observations made on these fungi by Mayen (1827), Corda (1842), Langenbeck (1845) and others. Carter, from 1859 to 1874, first clearly proved the pathogenic role of actinomyces in man, his work having been concentrated on Madura foot. The name "Actinomyces" (ray fungus) was introduced by the botanist Harz (1878) to describe the fungus granules which Bollinger (1877), discovered in a cow which was suffering with the disease known to veterinarians as "lumpy jaw." The extensive investigations of J. Israel and M. G. Wolff established the frequency of the disease in man and the identity of the infecting organism, now known as *A. bovis*, with that found by Bollinger in the cow. In 1890 Boestrom described cultures isolated from clinical cases of actinomycosis which contained organisms morphologically identified with the cultures of Israel and Wolff, but his cultures grew aerobically at room temperature. It is now known that many of Boestrom's cultures must have been saprophytic actinomyces from the soil, but some aerobic organisms of this type are pathogenic for animals and man. In the last years of the 19th and the early

30. Drake, C. H. and Henrici, A. T. 1943, *Nocardia asteroides*. Am. Rev. Tuberc. **48**: 184.

years of the 20th century, Vincent[31] Wright,[32] Musgrave, Clegg and Polk,[33] and Foulerton[34,35,36,37] added much to our knowledge of the aerobic type of actinomycosis, usually including both acid-fast and non-acid-fast organisms in their descriptions. Claypole[38] stated that the property of acid-fastness is variable and may be considered a function rather than quality, since it can be assumed or discarded by the organism apparently according to circumstances. Sanfelice[39] suggested that the lipid in those parts of the body normally associated with acid-fast organisms assists in developing this property. The use of carbolfuchsin as a stain is a further cause for confusion, as Goldsworthy,[15] Henrici and Gardner[2] and the authors discovered, because the degree of acid-fastness depends upon such variable factors as the length of time taken by the decolorization process, the concentration of the decolorizing agent, the source of the material stained, the mediums used for culture and the portions of the organisms stained. It would appear, therefore, that in recent years more emphasis than is justified has been placed on the acid-fast property for classification of the aerobic actinomyces, rather than relying on other characteristics, and that many of the aerobic organisms reported but not included in review by Henrici and Gardner and others could well have been *N. asteroides*. The acid-fastness of this organism, even at best, is much less than the tubercle bacillus; and although the acid-fast actinomycetes form a group which is not of great practical importance because infections caused by them are rare, they are of scientific interest since they form a distinct connecting link between the bacteria proper and the higher fungi. In fact Henrici and Gardner[2] thought they could arrange the various species of acid-fast actinomycetes in a fairly uniform series, with Eppinger's variety at one extremity establishing contact with the acid-fast bacteria which it resembles very closely in cultural characteristics, and their own strain at the opposite extremity where it joins the non-acid-fast actinomycetes. The confusion between tuberculosis and infections caused by acid-fast strains of actinomyces is well illustrated by many writers in their reports on pseudotuberculosis and pseudoactinomycosis.[39,40,41,42] In pulmonary disorders an acid-fast actinomyces infection is not easily differentiated from tuberculosis, and undoubtedly this infection has been diagnosed mistakenly as tuberculosis. In the sputum the organism readily undergoes fragmentation, and, being acid-fast, the fragments resemble closely tubercle bacilli. They are more variable in length, and sooner

31. Vincent, H. 1894, Etude sur le parasite du "Pied de Madura." Ann. Inst. Pasteur **8**: 129.
32. Wright, J. H. 1905, The biology of the microorganisms of actinomycosis. J. Med. Research **13**: 349.
33. Musgrave, W. E., Clegg, M. T. and Polk, M. 1908, Streptotrichosis, with special reference to the etiology and classification of Mycetoma. Phil. J. Sc. **3**: 477.
34. Foulerton, A. G. R. 1899, Streptothrix infections. Lancet **2**: 779.
35. Foulerton, A. G. R. 1906, Pathology of *Streptothrix* infections. Lancet **1**: 970.
36. Foulerton, A. G. R. 1910, Streptothricosis and tuberculosis. Lancet **1**: 551; 626; 769.
37. Foulerton, A. G. R. 1913, Some observations on a series of 78 cases of *Streptothrix* infection. Lancet **184**: 381.
38. Claypole, E. 1914, Human streptotrichosis and its differentiation from tuberculosis. Arch. Int. Med. **14**: 104.
39. Sanfelice, F. 1905, Streptothrix-Pseudotuberculose. Centralbl. f. Bakt. **38**: 30.
40. Berestneff, N. 1898, Ueber Pseudo-Aktinomykose. Ztschr. f. Hyg. **29**: 94.
41. Flexner, S. 1898, Pseudo-tuberculosis hominis streptothrica. J. Exper. Med. **3**: 435.
42. Gair, G. 1917, Streptotrichosis or pseudoactinomycosis in man (pseudo-tuberculosis). Vet. J. **73**: 96.

or later long branched filaments are found.

In 1881 Eppinger[43] described an aerobic, acid-fast actinomyces in a brain abscess and described it under the name of *Cladothrix asteroides*. One of the earliest descriptions of these acid-fast types was by Nocard[44] in 1888, when he described an acid-fast strain in farcin du boeuf, a disease in cattle suspected of being tuberculosis until Nocard clearly demonstrated that the organism was an acid-fast actinomyces. The acid-fast property was not recognized by various bacteriologists who first described these organisms and was first observed apparently by Berestneff[45] in 1897. Feistmantel[46] in 1902, after careful experiments and critical observation of *Streptothrix nocardi* (*farcinica*) as to its acid-fastness and variation in structure in tissues, asserted its close relation to the acid-fast organisms on one side and actinomyces on the other, calling them all *Streptothrix*. These aerobic, variably acid-fast organisms have since been designated as *Streptothrix eppingeri*, *Streptothrix asteroides*, *Oospora asteroides*, *Actinomyces asteroides*, and at the present time are termed *Nocardia asteroides*, as mentioned.

43. Eppinger, H. 1890, Ueber eine neue pathogene Cladothrix und eine durch sie hervorgerufene Pseudotuberculosis. Beitr. z. path. Anat. u. z. allg. Path. **9**: 287.
44. Nocard, E. 1888, Note sur la maladie des boeufs de la Guadeloup. Ann. Inst. Pasteur **2**: 293.
45. Berestneff, N. 1897, Dissertation, Moscow, quoted by Schabad, 1904, Ztschr. f. Hyg. u. Infectionskr. **47**: 41.
46. Feistmantel, C. 1902, Saure- und Alkohol-Festigkeit der Streptothrix farcinica und die Beziehungen der Streptotricheen zu den Saurefesten Pilzen. Centralbl. f. Bakt. I, **31**: 433.

SUMMARY

Infections in man due to the aerobic, variably acid-fast *Nocardia asteroides* are rare as compared to those due to the anaerobic *Actinomyces bovis*. Thirty-seven cases of infection with *N. asteroides* have been collected from the literature. The mortality rate appears high: only 5 of the 37 cases could be considered as having recovered, and all 5 patients received sulfonamide therapy in addition to other forms of treatment. The infection was pulmonary in 33 cases, and metastatic abscesses of the brain were present in at least 11. Three patients with nocardiosis due to *N. asteroides*, two of them terminating fatally, are reported. The organisms isolated from the lesions belong to the relatively rare aerobic, variably acid-fast group of actinomyces. Its characteristics are described in detail.

The acid-fastness of *N. asteroides* is a variable property. In distinguishing this group of organisms from other pathogenic actinomycetes, much more emphasis should be given to other properties, such as the readiness with which they may be cultivated on artificial mediums, their cultural characteristics, their virulence for laboratory animals, and the usual absence of "sulfur granules" and "club-formation" at the periphery of the mycelial masses. The importance of the actinomycotic infections lies in the possibility of their being confused with tuberculosis. Nocardiosis should be considered in all patients with obscure or unusual chronic infections, especially when repeated attempts to demonstrate the tubercle bacillus have failed. The treatment of choice in the aerobic type of actinomycotic infection appears to be sulfadiazine or other sulfonamides, rather than antibiotics such as penicillin and streptomycin.

ENTERIC ORGANISMS FROM THE AMERICAN COCKROACH

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The significance of certain arthropods as vectors of disease has long been firmly established. In the case of the cockroach, although there have sometimes been good grounds for suspicion, proof of a definite part in disease transmission has been lacking.

Roaches may be found wherever organic material and moisture are present. In temperate and tropical regions they inhabit refuse piles, latrines, industrial plants, food stores, hotels, restaurants, and hospitals, and are one of the major pests of the housekeeper. They are numerous in and around the cages of laboratory animals unless constant preventive measures are practiced. It is probable that they live in close association with such animals as rats and mice in the wild state. In view of their feeding habits, the possibility of their becoming infected or acting as carriers of a variety of micro-organisms is clearly evident.

Five species of roaches, world-wide in distribution, are found in this country. These include the small German roach or "croton bug," *Blatella germanica*, the larger Oriental roach, *Blatta orientalis*, the Australian roach, *Periplaneta australasiae*, the tropical roach, *Supella supellectilium*, and the large winged American cockroach, *Periplaneta americana*.

Cao,[1] in 1898, was the first to report on the natural flora of the roach. He isolated *Bacillus megatherium*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Pseudomonas non-liquefaciens* from *Blatta orientalis*. He was also able to recover each of several pathogenic types after feeding with the respective organism. Morrell,[2] in 1911, cultured the feces of 17 specimens of *Blatella germanica* on shipboard, where there was no access to putrescent material. From 12 of these, no gram-negative rods were isolated. The remaining 5 specimens yielded *Bacterium lactis aerogenes* and *Bacterium cloacae*. Morrell likewise was able to feed and recover several pathogenic organisms. Among other investigators who have fed and recovered pathogenic bacteria from one or more of the roach species may be mentioned Longfellow,[3] Morischila and Tsuchimochi,[4] Spinelli and Reitano,[5] Tejera,[6] Barber,[7] Toda,[8] and Macfie.[9]

In 1915, Scott,[10] studying the epidemiology of enteric fever in Kingston, Jamaica, concluded without experimental proof that roaches were con-

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1. Cao, G. 1898, Sul passagio dei microorganismi a traverso l'intestino di alcuni insecti. Uff. San. Riv. Igiene Med. Pat. **11**: 385–397.
2. Morrell, C. C. 1911, Bacteriology of the cockroach. Brit. M. J. **2**: 1531.
3. Longfellow, R. C. 1913, The common house roach as a carrier of disease. Am. J. Pub. Health **3**: 58–61.
4. Morischila and Tsuchimochi, 1938, cited in Medical Entomology, ed. 2, by W. A. Riley and O. A. Johanssen. New York, McGraw-Hill Book Co., Inc.
5. Spinelli, A. and Reitano, U. 1932, Ricerche sulle blatte, quali agenti di diffusione dei germi del colera, della febbre tifoide e della dissenteria bacillare. Ann. d'ig. **42**: 745–755.
6. Tejera, E. 1926, Les blattes envisagée comme agentes de dissémination des germes pathogènes. Compt. rend. Soc. de biol. **95**: 1382–1384.
7. Barber, M. A. 1914, Cockroaches and ants as carriers of the vibrios of Asiatic cholera. Philippine J. Sc. (Ser. B) **9**: 1–4.
8. Toda, T. 1923, Cholera and the ship cockroach, J. Hyg. **21**: 359–361.
9. Macfie, J. W. S. 1922, Observations on the role of cockroaches in disease. Ann. Trop Med. **46**: 441–448.
10. Scott, H. H. 1915, An investigation into the causes of the prevalence of enteric fever in Kingston, Jamaica, with special reference to question of unrecognized carriers. Ann. Trop. Med. **9**: 249.

cerned in the spread of the disease.

At Viterbo, Italy, in 1930, Antonelli[11] made a study of two small outbreaks of typhoid fever. Water was uncontaminated, flies were absent, and there was no known contact among members of the affected families. Examination was made of *Blatta orientalis* from the open latrines of the affected families, which were located near each other. Typhoid bacilli were recovered from the feet and bodies of these roaches and were found to multiply on bread and cheese on which the insects were allowed to deposit feces.

Beck and Coffee,[12] in 1943, reported an interesting case of probable transmission under natural conditions from roach to animal of a pathogenic organism. The guinea pig colony of the Texas State Department of Health had suffered heavy losses due to *Salmonella typhimurium* infection. The organism was recovered from both the appendages and the intestines of *Periplaneta americana* infesting the cages. *Blatella germanica* from the same cages were negative for the organism. In this instance, as well as in the work of Antonelli cited above, it will be noted that the roaches were cultured only after cases of the disease had occurred.

The status of the role of the cockroach in disease transmission was summarized by Patton[13] in 1930 as follows:

> . . . it is necessary to point out that although there is ample evidence that certain pathogenic organisms when fed to the cockroach can be recovered from its feces, there is *no proof* that under natural conditions the cockroach becomes infected and then infects man's food.
>
> Until pathogenic organisms have been recovered from the feces of cockroaches living under natural conditions . . . and the findings confirmed by a bacteriologist, the cockroach can, like many other household insects, be regarded *only* with suspicion.

The investigation here reported has been concerned with a search for members of the enteric group of bacteria present under natural conditions in the digestive tract of *Periplaneta americana*, a roach which is readily available in Texas. The material cultured consisted of the contents of the hind gut, obtained by dissection. It seems probable that the bacteria which can be isolated from the hind gut contents will not be significantly different from those which would be found in freshly deposited feces of the same insect. The results should, therefore, be an index to the type of contamination introduced into materials soiled with roach feces.

SOURCES OF INSECTS

Cockroaches from Austin, Texas, and San Antonio, Texas, were used for study. The Austin series included roaches captured during the months of September, October, and November, 1947, at a time when human health conditions in the city might be considered normal. These were collected chiefly on the kitchen porch of the nurses' home annex of a hospital. A few were collected on the entrance porch of the hospital, some were captured in the hospital laboratory, and several were obtained from a private home. The usual method of capture was to place a Petri dish cover over the insect, slip a piece of paper under roach and cover, and transfer to a wide-mouth jar. A total of 53 adult roaches were examined in this series.

In the San Antonio series 41 insects, including 29 adults, 6 large nymphs, and 6 small nymphs, were examined. These were collected chiefly at sewer manholes in two widely separated areas of the city, through the courtesy of members of the Sanitary Engineering Division of the San Antonio Health Department. Two of the roaches were captured in a private home. Roaches in this series were examined during the first two weeks of June, 1948, in a period of excessive enteric disease among infants. About 25% of the stool cultures of affected infants, tested by the City Health Department, yielded *Shigella sonnei* and *Shigella paradysenteriae*.

METHODS

The roaches were lightly chloroformed. Wings were clipped off near the base and the insects were soaked in alcohol for about 5 minutes.

11. Antonelli, G. 1930, La blatta nella igiene domestica. Gior. d. r. Soc. ital. d'ig. **52**: 132–142.
12. Beck, O. and Coffee, W. B. 1943, Observations on *Salmonella typhimurium*. J. Bact. **45**: 200.
13. Patton, W. S. 1931, Insects, Ticks, Mites, and Venomous animals, Croydon, H. R. Grubb, Ltd.

They were removed from the alcohol with sterile forceps and placed, dorsum uppermost, on a pad of sterile gauze. With small, pointed scissors, kept hot enough to sear, the dorsum was clipped at the side and posterior edges and laid back, exposing the intestinal tract. The intestine was lifted with a teasing needle and small forceps, and the hind portion clipped out with scissors. The part removed was macerated in broth on a sterile watch glass. The material was then taken up into a sterile dropping pipette and divided between two small test tubes containing, respectively, 5 ml of Selenite F broth and 5 ml of tetrathionate broth. Results indicated no superiority of one medium over the other, and with the San Antonio series only the Selenite F medium was used for enrichment. Dissections were done at night in a closed laboratory to minimize air-borne contamination.

The cultural procedures followed were those routinely employed in diagnostic laboratories. Plating mediums used were SS (Salmonella-Shigella), bismuth sulphite, and MacConkey's agar. These were streaked after 24 and 48 hour incubation of the enrichment cultures and were held for 72 hours with daily examination. The various colony types were picked to Kligler's medium and to plain nutrient agar as screening mediums. In the Austin series the coliform group was identified by means of IMViC tests, carbohydrate fermentation, and gelatine liquefaction. In the San Antonio series all rapid lactose fermenters were classified as "coliform," with no attempt to determine species.

Urea decomposition within 24 hours in the medium of Roland, Bourbon and Szturm[14] and spreading growth on the moist surface of nutrient agar slants were used as criteria for segregation of the *Proteus* strains. Cultures which failed to ferment dextrose, lactose, and sucrose were classified as *Alcaligenes*. Isolates which were negative or slow lactose fermenters and had not been classified as *Proteus* or *Alcaligenes* were sent to the Army Area Laboratory at Fort Sam Houston, San Antonio, for additional cultural tests and serological typing where indicated.*

RESULTS

In the Austin series, 9 of the 53 insects were negative for enteric bacteria, 36 were positive for members of the coliform group, 8 for *Alcaligenes fecalis*, 7 for *Proteus*, 1 for *Eberthella oedematiens*, and 1 for *Shigella alkalescens*. A single species was isolated from 29 of the roaches, 9 yielded 2 species, 3 were positive for 3 species, 2 for 4 species, and 1 for 5 different species including an *Escherichia*, an *Aerobacter*, an *Alcaligenes*, and 2 species of *Proteus*.

As is customary in identification studies of gram-negative non-spore-forming rods, several of the isolates were irregular in that they did not agree in one or more particulars with the characteristics of the species which they resembled most closely. Where the discrepancy was of minor significance the irregular strain was assigned to the species which it most closely resembled. There were 7 coliforms and 2 *Proteus* cultures which were too irregular to allow assignment to a species.

The frequency of occurrence of the various types isolated is as follows: *Escherichia coli* 20, *Escherichia freundi* 2, *Escherichia intermedium* 1, *Aerobacter aerogenes* 7, *Aerobacter cloacae* 14, *Aerobacter* sp. 1, *Paracolobactrum aerogenoides* 2, *Paracolobactrum coliforme* 2, irregular coliforms 6, *Proteus morganii* 2, *Proteus mirabilis* 3, *Proteus rettgeri* 1, *Proteus vulgaris* 1, *Proteus* sp. 2, *Alcaligenes fecalis* 8, *Shigella alkalescens* 1, *Eberthella oedematiens* 1.

Gram-negative rods were isolated from all roaches of the San Antonio series. In this series no identification to species was made of the rapid lactose fermenters or of the *Proteus* group. The frequency of occurrence of the types isolated is as follows: Coliform group 18, *Proteus* group 37, *Pseudomonas* sp. (probably *Ps. aeruginosa*) 1, *Salmonella schottmuelleri* 2, *Salmonella oranienburg* 1, *Salmonella bredeney* 1.

One of these roaches yielded as many as three non-lactose-fermenters. Another yielded four, including *S. schottmuelleri*, *S. bredeney*, *A. faecalis*, and a *Proteus*.

14. Roland, F., Bourbon, D. and Szturm, S. 1947, Différenciation rapide des entérobactériacées sans action sur le lactose. Ann. Inst. Pasteur **73**: 914–916.

* The cultural and serological tests at the Army Area Laboratory were done through the co-operation of Colonel Dwight M. Kuhns and Mrs. Dorothy Anderson. Two strains of *Salmonella* difficult to classify were identified by Dr. D. W. Bruner of the University of Kentucky.

No significant difference was found between the flora of nymphs and adults.

DISCUSSION

As negative results were obtained from 9 insects in the Austin series, it would appear that enteric bacteria are not constant inhabitants of the gut of the roach, but are of irregular occurrence therein. It will be noted that *Proteus* species were found abundantly in the June series of roaches, and were less abundant during the fall months; they had disappeared completely by November. Several investigators have reported seasonal variation in the bacterial flora of insects. The flora may serve as an index to the food and environment of the insect.

As there was no correlation between organisms isolated from the San Antonio roaches and organisms isolated from stools of infants suffering from diarrhea, no evidence was obtained to connect roaches with the spread of the epidemic. However, three known pathogens, the *Salmonella* species, were isolated from these roaches.

Organisms of doubtful pathogenicity isolated from the combined series included *Proteus* strains, *Alcaligenes fecalis Pseudomonas aeruginosa*, *Shigella alkalescens*, and slow lactose coliforms. All of these organisms have been implicated in the causation of disease where no other possible pathogen was recovered. The fact that they frequently occur in the absence of disease does not eliminate them as pathogens, as the carrier state is common for well established disease-producers of the enteric group. Several investigators suggest that these organisms may lack invasiveness and that a predisposing factor may be needed before they can act as pathogens. Worrall[15] and others hold the view that infant diarrhea is frequently due to infection with organisms not ordinarily pathogenic for adults.

Because of the lack of conclusive evidence that roaches can transmit disease, these insects have been regarded with tolerance by a large proportion of the population, especially in areas where roach control is difficult.

The results cited above suggest that such tolerance is unwarranted and that every effort should be made to suppress the cockroach and to protect food and kitchen utensils from contact with its feces. Roach control is clearly of the highest importance in households that include infants.

SUMMARY

Cultures for enteric organisms were made of the hind gut contents of 94 specimens of the American cockroach, *Periplaneta americana*. Three pathogenic species were isolated: *Salmonella schottmuelleri*, *Salmonella oranienburg* and *Salmonella bredeney*. Organisms of doubtful pathogenicity recovered included *Proteus vulgaris*, *Proteus morganii*, *Proteus mirabilis*, *Proteus rettgeri*, *Paracolobactrum* sp., *Alcaligenes fecalis*, and *Pseudomonas aeruginosa*. Non-pathogenic isolates included *Escherichia* and *Aerobacter* species and *Eberthella oedematiens*.

Since this manuscript was submitted for publication, there has come to the attention of the authors the report by Mackerras and Mackerras (1948, Australian J. Sc. **10**: 115) of the recovery in three instances of *Salmonella bovis-morbificans* from *Periplaneta americana*, and of *Salmonella typhimurium* in one instance from *Nauphoeta cinerea*. The roaches examined by these investigators were captured in hospital wards where cases of gastroenteritis due to *Salmonella bovis-morbificans* were occurring. Mackerras and Pope (1948, Australian J. Exper. Biol. & M. Sc. **26**: 465–470) were able to infect experimentally 4 species of cockroaches with 5 species of *Salmonella* and found viable *Salmonella* present in roach feces for as long as 40 days from infection.

15. Worrall, R. L. 1944, Diarrhaea and vomiting of infants. Brit. M. J. **2**: 865.

INDUCED RESISTANCE TO BACITRACIN IN CULTURES OF *STAPHYLOCOCCUS AUREUS*

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The present study is concerned with the rate and extent of increase and decrease in resistance to bacitracin by four strains of *Staphylococcus aureus*. Cross resistance between bacitracin and penicillin is also considered.

Induction of resistance to penicillin by originally susceptible strains of *Staph. aureus* has been shown to be rapid by many workers. Rammelkamp and Maxon,[1] McKee and Houck,[2] Bondi and Dietz,[3] Demerec,[4] Todd et al,[5] Luria,[6] Graessle and Frost,[7] Klimek et al,[8] and Bellamy and Klimek[9] found that resistance rose rapidly, and as a result of increased resistance to penicillin, strains of *Staph. aureus* developed morphological and cultural changes in their characteristics as well as a decrease in rate of growth.

Cultures of *Staph. aureus* that had become resistant to penicillin could be returned to their original range of sensitivity by subcultivation in plain broth (Todd et al,[10] Graessle and Frost,[7] Klimek et al,[8]) or by storage in dry ice (Graessle and Frost[7]). Resistance could be maintained for three months by keeping the culture in the refrigerator or on agar slants (Demerec[4]).

Graessle and Frost[7] found that penicillin-resistant cultures showed a slight cross resistance to streptomycin but the reverse did not occur. Klimek et al[8] showed slight cross resistance of penicillin-resistant staphylococci to streptomycin and of streptomycin-resistant staphylococci to penicillin.

MATERIALS AND METHODS

Test organisms.—Four strains of *Staph. aureus* were employed. They were the FDA strain #209, the Oxford strain obtained from the American Type Culture Collection, L strain (isolated from a patient with a chronic mastoid infection), and strain H 109, which was originally obtained from Merck Laboratories of Rahway, New Jersey.

Antibiotics.—Crystalline penicillin G (Bristol) was used. The bacitracin was obtained through the courtesy of Ben Venue Laboratories. Dilutions of the antibiotics were made with sterile nutrient broth or sterile physiological sodium chloride solution.

Cultures.—Stock cultures were maintained in the refrigerator on nutrient agar slants and transferred monthly. Cultures used in assays were 18- to 24-hour nutrient broth cultures grown at 37 C. Antibiotic control cultures were prepared by inoculating 10 ml of nutrient broth with 0.1 ml of a preceding 48-hour broth subculture of the original parent strain. Test cultures for developing resistance were prepared by inoculating 10 ml of broth with 0.5 ml of the end point tubes from the previous titrations. All cultures, with a few

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Part of a thesis submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1. Rammelkamp, C. H. and Maxon, T. 1942, Proc. Soc. Exper. Biol. & Med. **51**: 386–389.
2. McKee, C. M. and Houck, C. L. 1943, Proc. Soc. Exper. Biol. & Med. **53**: 33–34.
3. Bondi, A., Jr. and Dietz, C. C. 1944, Proc. Soc. Exper. Biol. & Med. **56**: 135–137.
4. Demerec, M. 1945, Proc. Nat. Acad. Sc. **31**: 16–24.
5. Todd, E. W., Turner, G. S. and Drew, L. G. W. 1945, Brit. M. J. **1**: 111–113.
6. Luria, S. E. 1946, Proc. Soc. Exper. Biol. & Med. **61**: 46–51.
7. Graessle, O. E. and Frost, B. M. 1946, Proc. Soc. Exper. Biol. & Med. **63**: 171–175.
8. Klimek, J. W., Cavallito, C. J. and Bailey, J. H. 1948, J. Bact. **55**: 139–145.
9. Ballamy, W. D. and Klimek, J. W. 1948, J. Bact. **55**: 153–160.
10. Todd, E. W., Turner, G. S., and Drew, L. G. W. 1945, Brit. M. J. **2**: 603–604.

exceptions, were diluted 1 to 500 in broth for use in bacitracin and penicillin assays. It was estimated by plate counts that from ½ to 1½ million bacteria, varying with the strain, were added to the assay tubes as the inoculum. It was observed that when undiluted inoculums were used, growth was obtained in higher concentrations of bacitracin than when the cultures were diluted as stated above.

Determination of inhibitory end points of the antibiotic.—The method of titrating bacitracin consisted of a tube dilution technique. Dilutions were prepared in ranges of 0.01 unit per ml to 0.1 unit per ml in hundredths, 0.1 unit per ml to 1.0 unit per ml in tenths, and from 1.0 unit per ml to 10.0 units per ml in whole units. Higher concentrations were used as necessary, and dilutions between the usual quantities were occasionally set up, for example, 1 to 2 in tenths, 0.1 to 0.2 in hundredths, 2 to 3 in tenths, etc. The tubes were prepared so that there were 1.9 ml of the mixture of broth and the antibiotic in proper concentrations. One-tenth ml of the diluted culture was then added, bringing the final amount up to 2.0 ml. The end point tube was that tube containing the least amount of antibiotic and causing complete inhibition of the test organism after 24 hours at 37 C. It was noted that the length of time of incubation affected the end point since a higher end point was obtained after 48 hours' incubation. A culture control tube containing 1.9 ml of the medium employed and 0.1 ml of the diluted culture accompanied the titration of each test organism. Bacitracin controls were run daily, titrating the parent subculture simultaneously with the titration of the resistant variant from the same strain.

Method of increasing resistance.—The technique accepted as the most suitable for this study as a result of preliminary observations was the transfer of 0.5 ml from the end point tube to 10 ml of nutrient broth. All such tubes were incubated at 37 C for 18 to 24 hours, and growth was almost invariably obtained. At the same time 0.1 ml transfers were made to 10 ml of broth from 48-hour broth subcultures of the original parent strains to be used as antibiotic controls. Titrations were then made on the following day using these cultures as the test organisms, and 0.5 ml of the end point tube, now containing a higher concentration of the antibiotic, were again transferred to 10 ml of nutrient broth. The assays were set up so that there were 1 to 3 tubes below and 1 to 3 tubes above the previous end point, depending upon the circumstances.

Method of decreasing resistance.—Resistant cultures were treated in two ways for returning them to their original susceptibility to the antibiotic. These methods consisted of maintaining broth cultures of the resistant variants in the refrigerator and of daily transfers in plain nutrient broth. Assays were made at appropriate intervals for possible loss of resistance.

Determination of cross resistance.—Cross resistance or sensitivity was determined by titrating the strain that was resistant to one antibiotic against the other antibiotic by the standard method used in the other assays.

EXPERIMENTAL RESULTS

In the initial assays with bacitracin, strain FDA #209 had an end point of 0.09 per ml, whereas the three other strains had end points of 0.2 unit per ml.

Rise of resistance.—Against bacitracin the peaks of resistance were 80-fold for strain H 109, with a maximum of 63 exposures, 125-fold for strain 209 after 62 exposures, 3-fold for the Oxford strain in 35 exposures, and 20-fold with strain L in 40 exposures. The curve for increase in resistance to strain H 109 is shown in figure 1. This curve in its rise of resistance is representative of the others, with the exception of the Oxford strain which showed no appreciable rise in resistance to bacitracin, and indicates that there was a steady rise despite some fluctuation. It appears that at approximately 50 times the original end point there is a tendency for the curve to level off, followed by a much slower rise in resistance.

Decrease in resistance.—The decrease in resistance to bacitracin was studied for strains H 109 and 209. By transferring the resistant cultures daily in plain nutrient broth, there was a rapid drop during the first 7 transfers. Subsequently the decrease continued at a much slower rate. The resistant cultures of the same strains, when maintained in the refrigerator in nutrient broth, showed only a very slight or no decrease in resistance after 29 days of storage. A representative curve for decrease in resistance is

indicated in figure 1. There was no drop in resistance to bacitracin after maintenance of the broth culture in the refrigerator for 29 days. After 7 and 8 transfers in bacitracin-free nutrient broth, there was a decrease in resistance from 40 times the original end point to 10 times the original end point. Following this initial decrease, there was a slower drop to 8 and 6 times the original resistance in 14 and 21 transfers, respectively. After 29 transfers the resistance still remained 6 times its original level.

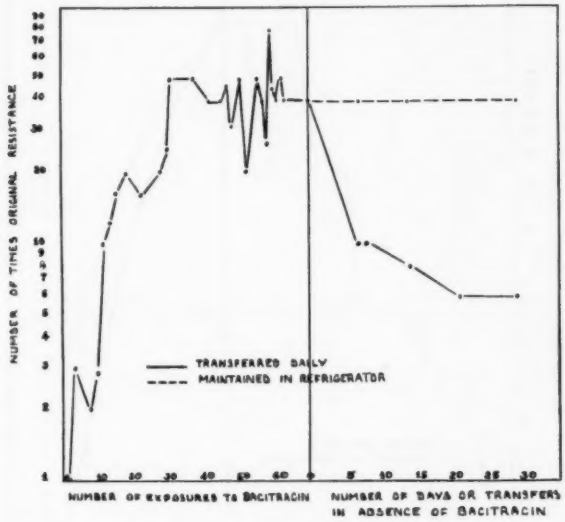
NUMBER OF TIMES ORIGINAL RESISTANCE
TRANSFERRED DAILY
MAINTAINED IN REFRIGERATOR
NUMBER OF EXPOSURES TO BACITRACIN
NUMBER OF DAYS OR TRANSFERS IN ABSENCE OF BACITRACIN

FIG. 1.—Rise and fall in resistance to bacitracin by *Staph. aureus* H 109.

Cross resistance.—For this study, resistance to penicillin was also induced in the four strains. Figure 2 shows the representative rise and fall of resistance to penicillin. As the resistance to bacitracin rose, there was no corresponding rise in resistance to penicillin, but instead there appeared to be a slight drop in resistance to penicillin for two out of three strains. When the bacitracin resistance of strain H 109 was between 32 and 50 times its original, the penicillin titers ran between 0.02 and 0.03 unit per ml, while the titers of the parent strains were in the range of 0.04 and 0.08 unit per ml. For strain L the difference in penicillin titers was less marked, since the test assays gave end points ranging from 0.05 to 0.07 unit per ml, while the control showed end points of 0.07 and 0.08. This indicates a greater susceptibility to penicillin by the bacitracin-resistant variant of strains H 109 and L. There was, however, no indication of either increase or decrease of penicillin resistance accompanying increased resistance to bacitracin by strain 209. In a range of bacitracin resistance from 50 to over 125 times the original, all assays with penicillin gave end points for this strain of bacitracin-resistant cultures that were identical with the control cultures that had had no exposure to bacitracin.

The penicillin-resistant cultures gave evidence of being markedly altered in bacitracin susceptibility. As the resist-

ance to penicillin increased, there appeared to be simultaneously greater susceptibility to bacitracin. As the penicillin resistance returned toward normal, there was a concomitant return toward normal of the bacitracin titers, with the exception of those cultures that were refrigerated. The penicillin-resistant cultures that were maintained in the refrigerator lost their resistance to penicillin nearly as rapidly as those that were transferred daily in plain nutrient broth; but the greater susceptibility to bacitracin appears to be a more permanent feature of the refrigerator-stored penicillin-resistant cultures since there was no accompanying rise toward the normal titer of bactiracin. This phenomenon occurred with all four strains studied and is represented in figure 3 by strain L. It can be seen that as penicillin resistance rose from the original resistance to 8⅓ times its original the bacitracin titer decreased from 0.2 unit per ml to 0.06 unit per ml. At 33⅓ times the original penicillin resistance, the bacitracin titer remained at 0.06 unit per ml. When the penicillin resistance rose to 50, the bacitracin titer decreased to 0.03 unit per ml; and as the resistance increased above the 100 and 150 marks, bacitracin assays showed end points of 0.02 and 0.01 unit per ml, respectively.

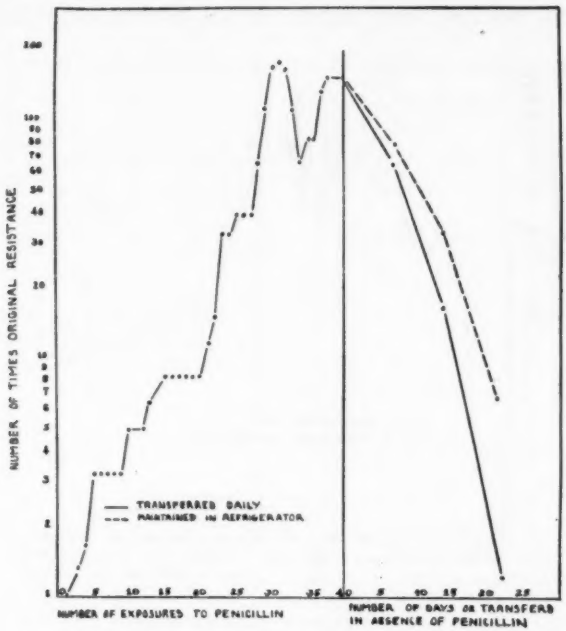
NUMBER OF TIMES ORIGINAL RESISTANCE
TRANSFERRED DAILY
MAINTAINED IN REFRIGERATOR
NUMBER OF EXPOSURES TO PENICILLIN
NUMBER OF DAYS OR TRANSFERS IN ABSENCE OF PENICILLIN

FIG. 2.—Rise and fall in resistance to penicillin by *Staph. aureus* strain L.

As the penicillin resistance was allowed to diminish by maintaining a broth culture of the resistant strain in the refrigerator, bacitracin end points remained at 0.01 unit per ml or less. In contrast, as the penicillin resistance decreased by daily transfers of the culture in penicillin-free broth, bacitra-

cin titers returned to their original and even rose above their original end points.

The greater susceptibility of bacitracin to penicillin-resistant cultures is not necessarily a specific phenomenon. Studies with other agents revealed that penicillin-resistant strains became more susceptible to these other agents in some instances. The penicillin-resistant variants of strains H 109, L, and 209 became between 3 and 7 times more susceptible to copper sulphate than the original control cultures of the same strains. Strain 209 became one-half as resistant to mercuric chloride as a result of increase in resistance to penicillin. The increase in susceptibility to bacitracin was on the order of 10 to 20 times for the corresponding cultures. Penicillin-resistant strain H 109 did not alter its susceptibility to mercuric chloride, and the Oxford strain showed no change in end point for either chemical despite its rise in resistance to penicillin and its greater susceptibility to bacitracin. The reason for this increased susceptibility of resistant variants may be due to a decreased rate of growth as suggested by Yegian, Budd and Middlebrook[11] for sulfonamide resistance and Bellamy and Klimek[9] for penicillin resistance.

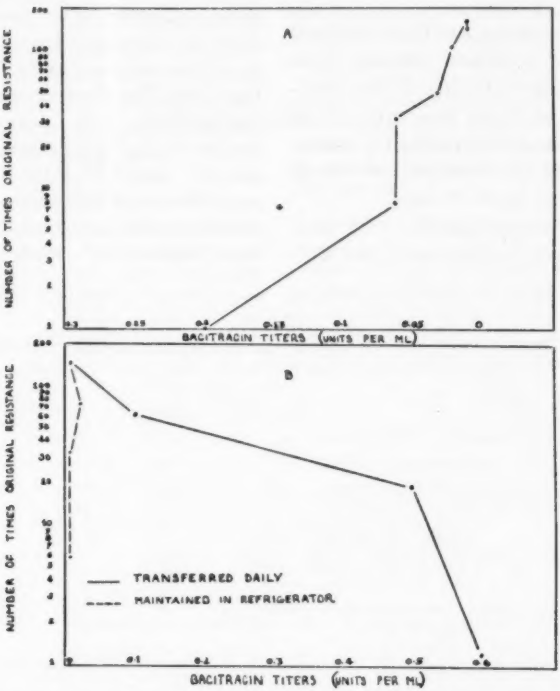

FIG. 3.—Effect of penicillin resistance on bacitracin titers of *Staph. aureus* strain L. A—Rise in penicillin resistance curve. B—Decrease in penicillin resistance curve.

11. Yegian, D., Budd, V. and Middlebrook, G. 1946, J. Bact. **51**: 479–485.

SUMMARY

1. The rises in resistance of four strains of *Staphylococcus aureus* to bacitracin were determined. Decrease in resistance was also studied.

2. Resistance to bacitracin rose irregularly but steadily, with a tendency to level off at about 50 to 60 times the original titer.

3. Loss of resistance to bacitracin was rapid if the resistant cultures were transferred daily in bacitracin-free nutrient broth, but there was little or no decrease in bacitracin resistance following storage of the resistant cultures in the refrigerator up to 29 days.

4. Cross-resistance studies with bacitracin-resistant variants indicated that there was either no change in penicillin resistance or a very slight decrease in resistance. Cross-resistance studies with penicillin-resistant strains revealed a greater bacitracin sensitivity as penicillin resistance rose; conversely, bacitracin resistance rose toward normal as penicillin resistance dropped. The rise toward normal of bacitracin resistance did not occur, however, when penicillin resistance was decreased by maintaining the penicillin-resistant cultures in the refrigerator. The phenomenon of increased susceptibility of penicillin-resistant strains to bacitracin was not entirely specific, since in some instances the penicillin-resistant cultures also showed an increased susceptibility to mercuric chloride and copper sulphate.

THE BEHAVIOR OF *PASTEURELLA PESTIS* IN GLYCERIN AND RHAMNOSE MEDIUMS

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Before discussing the results obtained when studying the behavior of 53 plague strains from the collection of the George Williams Hooper Foundation in glycerin and rhamnose mediums and comparing the findings with the observations of previous workers, it is necessary briefly to describe the technique used.

TECHNIQUE

Medium—One percent neopeptone dissolved in distilled water served as a base to which the fermentable substances to be tested were added in 1% proportions. Andrade's indicator was incorporated and the final pH of the medium adjusted to 7.40.

Procedure.—All cultures to be tested were first streaked out on blood agar plates to check for contamination. If found pure, loopfuls of the growths were transferred into the appropriate medium. The tubes were then incubated at 37 C and observed for 21 days. As recommended by de Smidt (1929), the growths were aerated by shaking the tubes daily. In order to make sure that this procedure kept the plague bacilli alive, subcultures on blood agar plates were made once weekly from each tube under test.

Sugar used.—In addition to glycerin and rhamnose, mediums containing glucose and lactose were used. Since *Pasteurella pestis* invariably acidifies glucose but causes no change in lactose, the two mediums mentioned last served as suitable controls.

RESULTS

The results obtained with the 53 strains mentioned above are summarized in table 1. The speed and the intensity of the acid production by 5 plague strains in glycerin or rhamnose are detailed in table 2.

TABLE 1.—*Behavior of 53 plague strains in glycerin and rhamnose mediums.*

Origin of strains	Number examined	Acid production in		Remarks
		Glycerin	Rhamnose	
California	26	None	1+ 25−	22 wild rodent, 2 human and 1 rat strain
Hawaii	10	None	1+ 9−	8 rat and 2 human strains
Palestine	2	None	None	These 2 strains from the San Francisco Public Health Service Laboratory formerly acidified glycerin.
Egypt	3	None	None	Human strains
Belgian Congo	1	+	None	Strain now avirulent
South Africa	2	None	None	Strains now avirulent
Madagascar	1	None	None	Girard's avirulent EV strain
India	2	None	None	Strains now avirulent
Java	5	1+ 4−	None	Strains now avirulent
Manchuria	1	+	None	Human strain, now avirulent

Studying the reactions produced by the plague bacillus in carbohydrate medium, Kaswashima (1934) distinguished three groups: strains which acidified neither glycerin nor rhamnose, those which produced acid in both cases and finally others which were acid in glycerin medium. As will be noted, our series comprises two strains falling into a 4th group, acidifying rhamnose but producing no change in glycerin medium.

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* Fellow of the American Bureau for Medical Aid to China, 1947–48 and Special Research Fellow, National Institute of Health, 1948–49.

It is clear that such a classification of *P. pestis* would be justified only if the reactions caused by the individual strains in glycerin and/or rhamnose medium remain stable. Francis (1932), when retesting in 1932 four differently derived subcultures of a ground squirrel strain originally examined in 1923, found no change in the fermentative reactions including those with glycerin and rhamnose, even though one of the subcultures had been subjected to 6 successive animal passages. Colas-Belcour (1926), retesting his plague as well as his pseudotuberculosis strains after one year, noted likewise no changes in the glycerin reactions. Korobkova (1936) found that 16 southeast Russian plague strains, when subcultivated after having kept for 10 to 12 years, at first failed to acidify glycerin but after further subcultivation once more became capable of doing so. Kurauchi (1936) confirmed that even repeated animal passages did not alter the behavior of his plague strains in glycerin medium and therefore considered this as stable in character.

However, some exceptional findings have to be noted: D'Aunoy, again examining in 1923 some of the strains tested by Wade in 1916, found that one of the formerly negative cultures acidified glycerin. In our own series of observations it was noted that (1) the two Palestine strains, which were said to have acidified glycerin in tests carried out about half a year previously in the San Francisco Plague Investigation Laboratory, failed to do so when retested by us and (2) although Otten's Tjiwidej strain which was kept in the Hooper Foundation for several years and another subculture of the same strain obtained from the Lister Institute (London) produced no change in glycerin medium, the culture "Tjiwidej 1948," recently received from Java, acidified such.

It cannot be said that the evidence regarding these aberrant reactions is fully convincing and it seems quite probable that further investigations will on the whole confirm the validity of Kurauchi's claim.

Colas-Belcour (1926) considered tests with glycerin medium as a suitable means to distinguish between *P. pestis* and pseudotubercle bacilli, which invariably produce acidity; an identical claim was made in regard to rhamnose by Bessonova and Lochow (1930). Even our limited observations disprove the differential diagnostic value of such tests. *P. pestis*, which may or may not affect glycerin and/or rhamnose, assumes thus a curiously intermediate position between pseudotubercle bacillus which always produces acidity in both mediums and *P. multocida*, which does not change them.

TABLE 2.—*Acid production in glycerin or rhamnose medium by 5 plague strains.*

Origin of strains	Name of strains	Acid production in	
		Glycerin	Rhamnose
California	B. 303	−	−(9), 4+(12)
Hawaii	193	−	−(10), 4+(11)
Java	Tjiwidej 1948	4+(21)	−
Belgian Congo	B.C. 343	−(4), 2+(1), 3+(1), 4+(15)	−
Manchuria	Harbin	−(2), 2+(1), 3+(1), 4+(17)	−

4+, 3+, 2+ and + indicate the extent of acid formation and − indicates no acid formation.
The number in parentheses indicates the duration in days of the reaction given before the parentheses. The reactions are listed in consecutive order of their occurrence.

DISCUSSION

In order to compare the findings

recorded above with those of previous observers, table 3 was prepared by consulting the available literature.

In a recently published paper Girard (1947) expressed the opinion that acidification of glycerin is peculiar to plague strains derived from wild rodents, whereas growths of rat or rat-flea origin produce no acid in the medium. Interesting though this hypothesis is, it must be noted that (1) all California strains were glycerin-negative, even though many of them were directly or indirectly of wild rodent origin; (2) only a minority of the South African cultures acidified glycerin, though plague in that area is mainly sylvatic; (3) most of the Argentine strains, a good part of which was no doubt derived from wild rodents, proved negative in glycerin medium.

Berlin and Borzenkov (1938) correlated the different behavior of plague growths in glycerin medium, not with the animal species from which the cultures had been derived, but with the localities from which they came. These workers distinguished between a glycerin-positive continental race of *P. pestis*, originating from the interior of Asia, and a glycerin-negative oceanic type, prevalent in the peninsulas and islands of the tropics. Kurauchi (1936), distinguishing between a glycerin-positive α type and a negative β type, had previously come to a similar conclusion.

It will be noted from table 3 that with one rather doubtful exception (a strain said to have come from Manchuria in D'Aunoy's series), all growths obtained from the central Asiatic plateau or its environs (i.e., from Manchuria, Mongolia, Transbaikalia, Turkestan, southeast and south Russia) belonged to the α type.

In marked contrast to these findings is that all or at least most cultures isolated in Japan, the Philippines, Hawaii, Indochina, Java, India, Ceylon, Madagascar, the Near East and Europe produced no acid in glycerin medium.

TABLE 3.—*Behavior of plague bacillus strains in glycerin reported by previous workers and reported in this study.*

Locality	Reference number	Strains examined	Glycerin-negative	Glycerin-positive	Remarks
Arabia	6	1	1	0	
Argentina	23	50	48	2	
Brasil	20	3	3	0	
California	7, 9, 16 and present study	63	63	0	14 human, 13 rat and 36 wild rodent strains
Ceylon	17	7	7	0	3 human and 4 rat strains
Belgian Congo	Present study	1	0	1	
Dutch East Indies	22 and present study	6	5	1	
Egypt	Present study	3	3	0	
England	8	35	35	0	Rat strains
France	5	1	1	0	
French West Africa	19	4	4	0	3 human and 1 rat strain
Greece	5	1	1	0	
Hawaii	Present study	10	10	0	2 human, 7 rat and 1 mouse strain
India	4, 6, 17, 20 and present study	8	8	0	
Indochina	5	1	1	0	
Italy	4	1	1	0	
Japan	13, 15	11	11	0	
Madagascar	5, 10, 12 and present study	5	5	0	Girard (1928) stated that other Madagascar strains were glycerin-negative.
Manchuria	6, 13, 16 and present study	36	1	35	
Mongolia	1	14	0	14	
Palestine	Present study	2	2	0	
Philippines	6, 24	9	7	2	
South Africa	18 and present study	24	20	4	
South Russia	2, 4	3	0	3	
Southeast Russia	2, 14, 19	148		148	
Southern United States	6, 24	28	28	0	12 human and 16 rat strains
Transbaikalia	4	6	0	6	
Turkestan	2, 4	22	0	22	
Total		503	265	238	

Modern plague outbreaks in these areas were due directly or indirectly to inroads of the infection from southeast China, which in turn was invaded from Burma via Yunnan Province. It is therefore likely that the Burmese, Yunnan and southeast China strains also belong to the glycerin-negative β type, but, as far as our information goes, no fermentation tests have been performed in these key areas. Until this gap in our knowledge is filled, it is impossible to gauge definitely the significance which the apparently regional distribution of the α and β types of *P. pestis* might have.

Possibly glycerin-positive strains are also prevalent in Central Africa, which, though probably not quite as ancient a home of plague as the central Asiatic plateau, also comprises endemic regions of long standing. However, all that is actually known is that a solitary strain from the Belgian Congo acidified glycerin and that 4 cultures isolated during one outbreak in a remote and recently invaded area of northwest Cape Province showed the same reaction. Further investigations of a sufficient number of strains from the African, particularly the central African plague foci, are therefore urgently called for.

Ways and means ought to be found as well to examine cultures from the various plague areas in South America, where thus far sufficient information is available from Argentina only.

SUMMARY

The results of a study of the reactions produced by 53 plague strains in glycerin and rhamnose mediums are reported and compared with the findings of previous observers.

As a rule, strains acidifying glycerin and those leaving such medium unchanged do not exist side by side in one and the same plague area, but either one or the other type is present or at least preponderant. Further concerted studies are necessary, however, before the possible significance of this distribution can be gauged.

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MUCOLYTIC ENZYME SYSTEMS.

VI. INHIBITION OF HYALURONIDASE BY SERUM IN INFECTIOUS DISEASES

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Previous investigations in this series on hyaluronidase inhibition by human blood serum disclosed elevations of the inhibitor level in patients with acute poliomyelitis,[1] various skin diseases of bacterial and virus origin,[2] and cancer.[3] The serum inhibitor levels in strains of mice susceptible to cancer[4] and in anaphylactically shocked normal and herpetic rabbits[5] were also studied. In the studies on infectious diseases, a marked correlation was found between the clinical condition of the animal or patient and hyaluronidase inhibitor level of the serum. Thompson[6] used the mucin clot prevention test to demonstrate an elevation of this blood factor in pneumonia, and Friou and Wenner[7] found a rise in rheumatic fever. Fulton et al,[8] who employed a method for the determination of hyaluronidase and hyaluronidase inhibitors depending on decapsulation of a group C hemolytic streptococcus, noted no difference in the degree of enzyme inhibition by the serums of normal individuals and patients with rheumatoid arthritis.

In order to determine how general the elevation of hyaluronidase inhibitor in serum is in infectious diseases, a number of cases from each of a variety of syndromes were studied. In some instances, repeated measurements on the same individual were made in order to correlate clinical condition and hyaluronidase inhibitor level. In others, only a single sample could be obtained, but this was taken at the height of the disease when possible. Comparisons of the temperature, erythrocyte sedimentation rate, and white blood cell count with hyaluronidase inhibitor levels were made with the data collected. In addition, the effects of vaccination with vaccinia virus were followed.

METHODS

The enzyme activity and its inhibition were determined by the viscosimetric method following the procedure previously given.[1] Bovine testicular enzyme and hyaluronic acid from hu-

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man umbilical cord were used. The inhibition was expressed as the quantity A, which is defined as $A=(R-R_0)/R_0$, where R_0 represents the time in seconds for the viscosity of the reaction mixture to fall to one-half its initial value, and R is the corresponding term for the fall in viscosity in the presence of serum. In the procedure employed, 0.02 ml of serum was required for each determination.

Rheumatic fever.—All of the cases studied with the exception of one, and possibly a second, had a low grade disease with little or no elevation of temperature and sedimentation rate, and their hyaluronidase inhibitor values were within the normal range. Only one case with relatively acute symptoms had a greatly elevated hyaluronidase inhibitor value. One case was followed over a period of time to observe changes accompanying recovery (see table 4). A steady decline of hyaluronidase inhibitor value was observed.

Rheumatoid arthritis.—The 17 patients investigated had chronic involvement of one or more joints, and all were receiving intravenous streptococcus vac-

penicillin and certain other antibiotics in serum had no effect on the hyaluronidase inhibitor values.[2] Three cases were followed in detail (see tables 1, 2, and 3). It is noteworthy that during the height of the disease the hyaluronidase inhibitor value of each patient was elevated, and as the clinical condition of the patient improved, the hyaluronidase inhibitor value declined.

TABLE 1.—*Case no. 1 of subacute bacterial endocarditis—male, age 36.*

Day after admission	2	9	15	16	23	29	33	51	52	78
A per ml		39	26		20	20		14		9.2
Temperature (high for day)	101.4	99.8	99.6	99.0	99.8	100.2		98.4	99.8	98.6
Sedimentation rate (mm in 60 min.)	88			78			104		80	61
Leucocyte count				14,700			8900			7700

TABLE 2.—*Case no. 2 of subacute bacterial endocarditis—male, age 31.*

Day after admission	1	2	11	15	16	17	21	32	39
A per ml		44	60	66		42	33		
Temperature (high for day)	104.0	104.6	104.0	99.0	98.6	98.4	98.6	99.2	98.6
Sedimentation rate (mm in 60 min.)							85	73	23
Leucocyte count	14,950	13,050	17,700		16,400		13,000	6,800	6,800

TABLE 3.—*Case no. 3 of subacute bacterial endocarditis—female, age 61.*

Day after admission	3	6	8	13	14	15	21	28	34	35
A per ml		46	52		43		25	21		17
Temperature (high for day)	102.0	100.2	99.6	99.0	99.0	99.0	98.2	99.2	98.6	98.2
Sedimentation rate (mm in 60 min.)	109			111			108		81	
Leucocyte count	8200					10,800	8300		9950	

RESULTS

Ninety serums from normal adults of all ages gave A values which had a mean of 16.2 with a standard deviation of 5.4.

Subacute bacterial endocarditis.—In the 6 cases of this disease studied, the hyaluronidase inhibitor level of the serum was uniformly increased at the height of the disease. In every case α hemolytic streptococcus was isolated from blood cultures on admission. All of these patients received massive dose penicillin therapy. They improved progressively and were discharged as apparent cures. It had been shown previously that the therapeutic levels of

cine therapy. These were out-patients and their temperatures were not recorded. Cases that were more acutely ill showed hyaluronidase inhibitor values that were distinctly elevated. Most of the patients were not acutely ill, and their inhibitor values did not deviate greatly from the normal.

Primary atypical pneumonia.—The three cases studied had elevated values. One case is presented in detail (table 5). The pattern of elevated hyaluronidase inhibitor values during the acute phase of the disease, dropping with recovery, is again demonstrated.

Infectious mononucleosis.—Fourteen cases of this disease with their respective heterophile antibody titers were studied. The range in values was large and the average value was not greatly elevated. No apparent correlation exists between the hyaluronidase inhibitor levels and temperatures or heterophile antibody titers in these patients.

Measles.—A case is presented in detail in table 6. The hyaluronidase inhibitor value which was high on the 6th day had fallen by the 10th, and on the 12th the patient developed a rising temperature and cough. Penicillin therapy was started. On the 14th day a rise in hyaluronidase inhibitor titer was found which may have been a reflection of the worsened clinical condition.

Other infectious diseases.—Elevated hyaluronidase inhibitor values were obtained in two out of three cases of brucellosis and in the cases of pertussis and scarlet fever (see table 7).

Vaccinia vaccination.—Normal males ranging in age from 17 to 29 were studied. The only element of selection introduced was that individuals who gave no history of previous vaccination

TABLE 4.—*Case of rheumatic fever—female, age 24.*

Day after admission	1	2	4	5	8	9	15	16
A per ml		52	48		27			16
Temperature (high for day)	98.6	101.2	100.6	98.6	98.0	98.0	98.0	97.8
Sedimentation rate (mm in 60 min.)	103			100		98	95	
Leucocyte count	8300					6350		

TABLE 5.—*Case of primary atypical pneumonia—female, age 44.*

Day after admission	4	7	9	16	20	21	22
A per ml			88	35	22		27
Temperature (high for day)	103.6	101.0	100.8	101.0	99.8	99.6	99.4
Sedimentation rate (mm in 60 min.)	51					87	
Leucocyte count	7400						
Cold agglutinin titer		1:448					

TABLE 6.—*Case of measles—female, age 29.*

Day after admission	6	7	10	12	13	14	21	23	27	30
A per ml	46		28			45	27	24	14	25
Temperature (high for day)	100.0	100.2	100.2	100.8	100.6	99.6	98.4	99.2	99.2	98.6
Sedimentation rate (mm in 60 min.)		80			93					
Leucocyte count		7300								

Penicillin was administered on the 12th day after admission.

were purposely included in order that the number of primary takes be as great as possible. In every one of the 12 primary takes the hyaluronidase inhibitor level in the serum rose between the 10th and 13th day after vaccination. It will be recalled that the maximum reaction in primary takes occurs in this period. In most cases this rise was at least double the normal value as measured by either prevaccination level or that to which the value fell after the reaction subsided. Accelerated reactors showed varied responses, and in the immune reactors the trend was toward no significant change (fig. 1).

The response obtained in individuals showing accelerated reactions reached a maximum in most cases earlier than the 10th to 13th day period of the primary takes. It is known that the maximum response in individuals showing accelerated reactions usually occurs before the 10th day, and it will be seen in figure 1 that in only one instance did the maximum response come later than 10 days after vaccination. The majority of individuals with immune reactions showed no significant change in hyaluronidase inhibitor level after vaccination, and in those cases in which changes were observed they followed no consistent pattern.

TABLE 7.—*Summary of data for the diseases studied.*

Disease	Number of cases	Age range (years)	Mean sedimentation rate(mm in 60 min.) and standard error of the mean	Mean A/ml and standard error of the mean
Subacute bacterial endocarditis	6	18–61	104.0± 4.8	44± 5.0
Rheumatic fever	11	16–56	31.9± 8.6	14± 3.9
Rheumatoid arthritis	17	27–80	60.8±13.9*	22± 5.1
Brucellosis	3	32–52		27± 5.6
Pertussis	1	27	35	39
Scarlet fever	1	24	11	28
Primary atypical pneumonia	3	20–44		48±16.4
Infectious mononucleosis†	14	17–32		19± 3.5
Measles	1	29		46
Normals	90	16–80		16± 0.6

* Mean of 5 cases.
† Heterophile titers ranged from 1:56 to 1:896. No correlation with A values noted.

Correlation of hyaluronidase inhibitor with sedimentation rate, temperature, and total leucocyte count.—A calculation of the coefficient of correlation for the hyaluronidase inhibitor in the serum compared to sedimentation rate, temperature, and total leucocyte count is given in table 8. A positive correlation with the sedimentation rate and temperature, which is significant as indicated by the data, and no significant correlation with the total leucocyte count was found. In the previous publication on skin diseases,[2] scatter graphs were included that were interpreted as indicating some correlation between the inhibitor and sedimentation rate, a closer correlation to temperature, and no correlation with leucocyte count. The present statistical treatment was applied to all of the data from both the previous paper[2] and this one, and it bears out the earlier interpretation with the exception that the correlation to

TABLE 8.—*Correlation of the hyaluronidase inhibitor values in blood serum with values of other measurements on the same patients.*

Measurement	Number of cases	Coefficient of correlation	K	P
Erythrocyte sedimentation rate	129	0.4	4.5	<0.001
Temperature	414	0.3	6.1	<0.001
Total leucocyte count	104	0.1	1.0	0.317

sedimentation rate ($r=0.4$) is actually a little better than to temperature ($r=0.3$).

DISCUSSION

The present data would seem to be in agreement with those previously obtained suggesting that the elevation of the hyaluronidase inhibitor in human blood serum is a general phenomenon in systemic infection which does not seem to be associated specifically with either bacterial or virus diseases or with particular syndromes in either group. The rather striking parallelism between the level of the serum inhibitor and the clinical condition of the patient is of particular interest, and the correlation between the inhibitor level and sedimentation rate and temperature serves to emphasize this parallelism. The syndrome that did not appear to fall into this category, at least from the data available, was infectious mononucleosis.

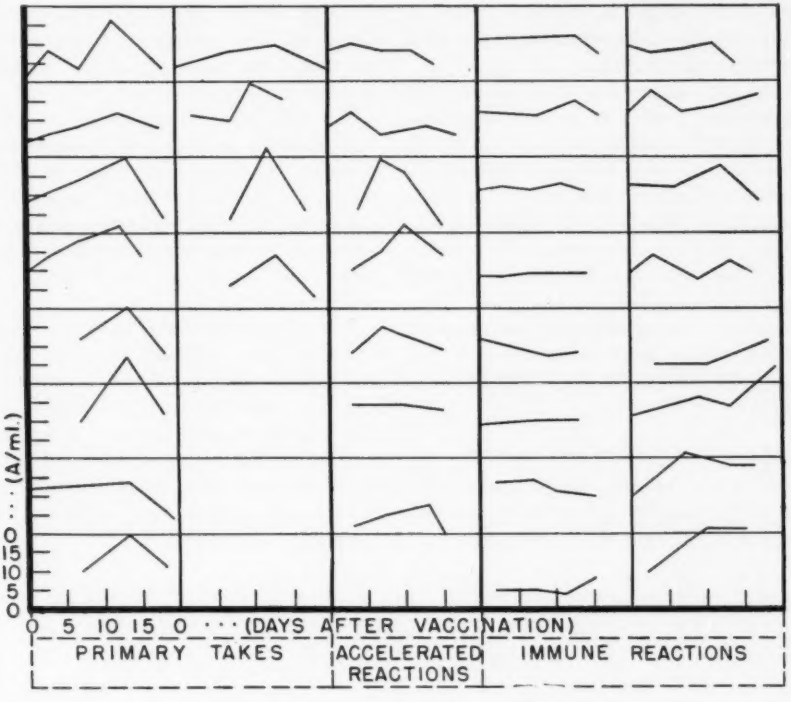

FIG. 1.—The response of the hyaluronidase inhibitor level in human blood serum to vaccinia vaccination.

The values in the rheumatic fever and rheumatoid arthritis groups were usually in the normal range because most of the cases were not sufficiently acute; the more acute cases were associated with elevated values.

The pattern of the responses of the serum inhibitor levels to vaccinia vaccination is largely that expected in the light of the other findings. The indi-

viduals exhibiting primary takes showed elevated levels whose magnitude paralleled the vaccination reaction itself. Varied responses were obtained with the accelerated reactors, and little if any change occurred in the immune reactors. Again, a correspondence was noted between the height of the reaction and the extent of the inhibitor elevation in those accelerated reactors with the more pronounced reactions.

As was previously emphasized,[4] the possibility cannot be ignored that the hyaluronidase inhibitor in blood serum may be a defense substance elaborated, at least in part, for the prevention of the spread of infection. Infective agents which do not in themselves contain hyaluronidase are nevertheless rendered more invasive by the enzyme. Conceivably, the non-specific elevation of the inhibitor level in both virus and bacterial infections may operate as a defense mechanism.

SUMMARY

A study of the hyaluronidase inhibitor in the blood serum of patients with infectious diseases was carried out. Six cases of subacute bacterial endocarditis, 11 of rheumatic fever, 17 of rheumatoid arthritis, 3 of brucellosis, 1 of pertussis, 1 of scarlet fever, 3 of primary atypical pneumonia, 14 of infectious mononucleosis, and 1 of measles were investigated and compared to 90 normals. With the exception of the infectious mononucleosis patients, elevations of the inhibitor levels which paralleled the severity of the disease were observed in all instances.

Statistical treatment of data revealed a significant correlation between the inhibitor level and erythrocyte sedimentation rate in 129 patients, between the inhibitor level and temperature in 414, but no significant correlation between the level and total leucocyte count in 104.

The changes found in the serum inhibitor level resulting from vaccinia vaccination were negligible in immune reactors, but definite elevations occurred in the cases of primary takes and in the accelerated reactors with the more pronounced reactions. The magnitude of these elevations paralleled the intensity of the reaction.

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